Thesis for the Master's degree in chemistry

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Approaches for characterizing unknown compounds by liquid chromatography in combination with mass spectrometry and nuclear magnetic resonance spectroscopy

60 study points

DEPARTMENT OF CHEMISTRY
Faculty of mathematics and natural sciences
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…stop and consider God´s wonders.

Job 37.14

Ask and it will be given to you;

seek and you will find;

knock and the door will be opened to you.

for everyone who asks receives;

he who seeks finds;

and to him who knocks,

the door will be opened.

Matt.7.7-8

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Preface

There are doors everywhere in many places. Most of them are closed unless someone opens a door or two. I am grateful to God that doors kept on opening up for me and would like to thank my supervisors, Prof. Elsa Lundanes, Prof. Frode Rise, Prof. Tyge Greibrokk, and Steven Ray Wilson, Ph.D., for giving me the opportunity to be part of their research projects and for tasks that challenged me to learn more throughout my studies. Thanks to their wise counsel and guidance, support and trust, this thesis of mine has now been completed. Special thanks further to Steven who truly helped me whenever a problem was encountered.

I would like to thank Dirk Petersen for his help at the NMR lab and Hege Lynne and her staff at the analytical course lab for their help and the use of the lab equipment. Thanks to Inge Mikalsen who fixed almost everything needed from time to time. Thanks to Leon Reubsaet and Håvard Loffheim for their help with the total protein measurement. Thanks to fellow students for the time we spent together including talks, sharing of ideas, and discussions.

Many thanks to my mother and family, Eva, Anna, Espen, and my friends, for their support, love, and for remembering me in their prayers. You make my life much easier.

Thank you, Long, for your love, support, and for believing that I managed to take good care of our children, Viet and Isabel, though at times I was quite overwhelmed with my studies. Studying is also part of the life and love that I always treasure for my children. Thank you.

Oslo, Norway, December 2007

Lan Thi Thu Nguyen

ABSTRACT

The stolen Scream painting of Edvard Munch was restored and in the restoration work of the damages on the painting, a glue of the materials which have to be somewhat identical to the glue that Munch used in the Scream painting was needed. A reference glue which was provided by the Munch museum and some origin materials from the Scream painting was analyzed in this thesis. The work presented characterized the two samples. The reference glue was analyzed by NMR with protein options. A tryptic digestion procedure with DTT recduction and IAM alkylation was explored in the study and the trypsination procedure was proved to function well in the method. The tryptic digested reference glue and the sample from the Scream were analyzed by LC-ESI-MS (TOF-MS and IT-MS). The protein and peptide identifications by Mascot search engine were also applied and discussed showing the identification procedures' pros and cons. The second part of this thesis, investigated some aspects for LC-SPE-NMR, such as DMSO as LC mobile phase, degassing effect on D_2O and CD_3CN and an $MgSO_4$ column was packed and investigated to see whether it could absorb water. Results from the testing showed that the water peak was retained, which is a seldom trait in chromatography. Though not tested further, a $MgSO_4$ column as a SPE column in the LC-SPE-NMR system was an idea which should be further.

ABBREVIATION

ACN	Acetonitrile
Beta-A	β-lactoglobulin-A
BSA	Bovine Serum Albumin
capLC	capillary Liquid Chromatography
CD3CN	deuterated ACN
Cyto-C	Cytochrome C
DMSO	dimetyl sulfoxide
DMSOd6	deuterated DMSO
D2O	deuterium oxide
DTT	DL-dithiothreitol
EIC	Extracted Ion Chromatogram
ES	electrospray
ESI	electrospray ionization
F.a.	formic acid
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single-Quantum Correlation
IAM or IAA	iodoacetamide
I.D.	Internal Diameter
IR	Infrared
IT	Ion Trap
LC	Liquid Chromatography
MP	mobile phase
MS	Mass Spectrometry
MS/MS	tandem MS/MS
MSDB	Mass Spectrometry protein sequence DataBase
m/z	mass to charge ratio
NCBI	National Center for Biotechnology Information
NMR	Nuclear Magnetic Resonance
S/N	Signal to Noise
SPE	Solid Phase Extration
TOF	Time-Of-Flight

Tris		trishydroxymethylaminomethan
3D		3-dimensional
2D		2-dimensional
UV		ultraviolet
Amino acids		
A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamate
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine

1. INTRODUCTION

1.1 The Scream painting by Edvard Munch

On 22nd of August 2004 *the Scream* painting by Edvard Munch was stolen from the Munch Museum, along with Madonna [1]. The Scream or *Skrik*, as it is named in Norwegian, is painted with a special technique known as tempera. Munch had painted the image directly on a cardboard which was again glued to a wooden board [2]. The Scream (1893) stolen from Munch museum is one of the two painted version of the worldwide known Scream image which is said to symbolize the mental image of the existential *angst* of civilised man [3]. The Scream has also become the key conception in the series of expressionist artworks by Edvard Munch. After two years of intense investigation, on 31st. August 2006 the Norwegian Police announced the recovery of the two stolen paintings (Figure 1) [4].

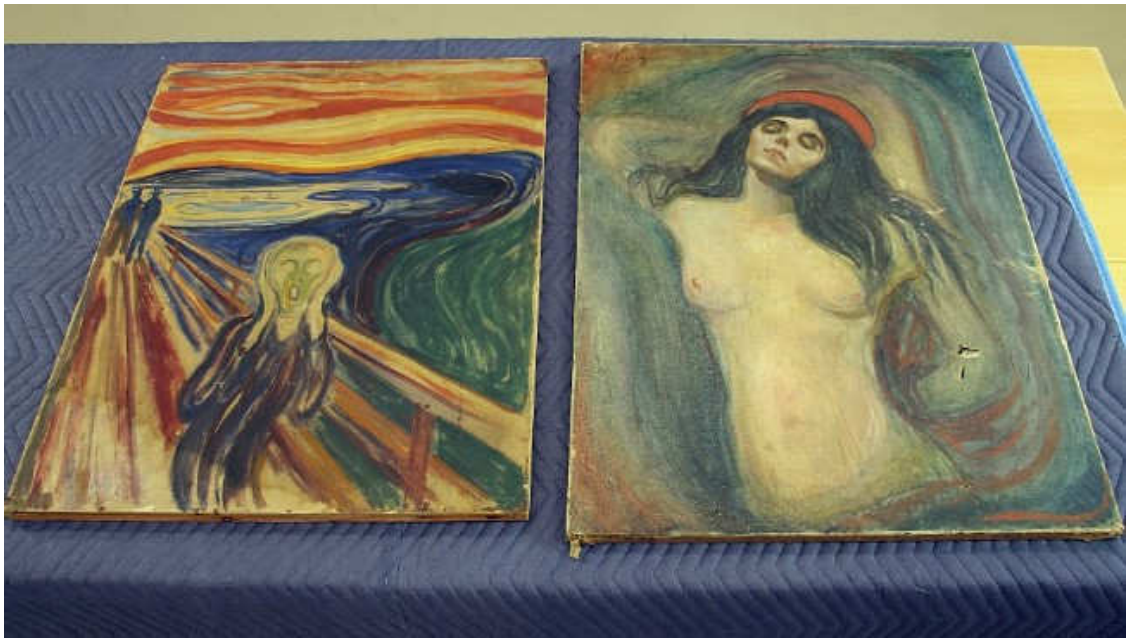


Figure 1: picture of the restored Scream painting on the left and Madonna on the right [4]

After examinations of the two paintings, The Scream showed damp damages in addition to rips on the paintings, and a comprehensive and time demanding restoration process including testing of chemical aspect of similar materials was considered necessary [2].

In restoration of a artwork it is necessary to know the chemical composition of the original materials and type of glue in use to define a proper program for the conservation in such a way that the integrity of the original materials and the characteritics of the painting materials is kept [5]. Tempera painting is often made by binding pigment in egg medium, sometimes along with other materials such as honey, water and milk in form of casein. Conservation treatments also make use of similar adhesives for fixing the flakes on painting surfaces.

The glue that Edvard Munch used in his artworks was suspected to be made from animal glue [6]. Animal glue is an adhesive created by prolonged boiling of animal connective tissues - animal skins, bones and tendons, and is formed through hydrolysis of the collagen from these animal tissues and can be produced from a variety of different animals e.g. horse, fish, rabbit [7]. These glues, and casein and egg based glues are all protein-based materials. When restoration of a painting is necessary, knowledge of the type of glue used is important for successful result. To reveal the type of glue used, analysis of the original glue is necessary. The sample has to be taken from the original painting layer, and is hence usually available in only a few micrograms in total, thus requiring sensitive analytic methods. Different application and identification of proteinaceous materials in paintings have been studied earlier with analytical methods such as High Performance Liquid Chromatography (HPLC) [8], Gas Chromatography-Mass Spectrometry (GC-MS) [9], MS [6], Time-resolved Fluorescence Spectroscopy [10] and Raman Spectroscopy [5].

Before representing the experimental work done in this study, the following give some basic information on the subjects.

1.2 Proteins

Proteins are large organic compounds made of amino acids. In the protein chain the amino acids are bond together by peptide bonds with a free carboxyl and amino group at the each end of the chain, the C-terminus and the N- terminus, respectively. A protein is a complete

biological molecule in a stable conformation and is measured with the number of amino acids and molecular mass (in Daltons (Da)). There are 20 amino acids which are directly encoded for protein synthesis by the standard genetic code. Naturally folded 3-dimensional (3D) protein is known as its native state shown in Figure 2, which shows three possible representations of the three-dimensional structure of the protein triose phosphate isomerase [11].

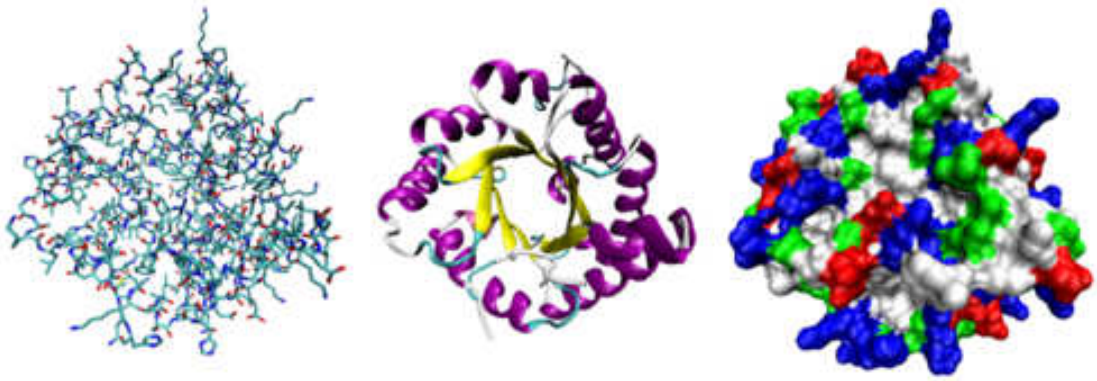


Figure 2: Three possible representations of the three-dimensional structure of the protein triose phosphate isomerase. Left: all-atom representation colored by atom type. Middle: simplified representation illustrating the backbone conformation, colored by secondary structure. Right: Solvent-accessible surface representation colored by residue type (acidic residues red, basic residues blue, polar residues green, nonpolar residues white).[11]

Other model structures of protein are also used such as primary structure - showing the amino acid sequence; secondary structure - showing the alpha helix and beta sheet with regularly repeating local structures stabilized by hydrogen bonds, (these contiguous segments of the protein is defined by specific bond angels in the polypeptide backbone), and the quaternary structure- showing the shape and structure resulting from interactions of other protein molecules. Hydrophobic, van der Waals, ionic, dipole, and hydrogen bonding are many of the forces that contribute to the formation of protein structures and influence how the proteins fold and interact with other molecules. Common experimental methods of protein structure determination are X-ray crystallography and NMR spectroscopy which can produce information at atomic resolution, and other methods such as cryoelectron microscopy and electron crystallography.

1.2.1 Peptides

A peptide consists of a relatively small and specific number of amino acids and often lacks naturally folded 3D structure which the protein has. Figure 3 in the following shows a peptide made of five amino acid residues linked together by the peptide bonds. The peptide bonds are formed by dehydration, removal of the water elements from the α-carboxyl group of one amino acid and the α-amino group of another. Peptides are named beginning with the amino-terminal residue. Peptides contain only one free α-amino group and one free α-carboxyl group, at the opposite ends of the chain. These groups ionize as they do in free amino acids.

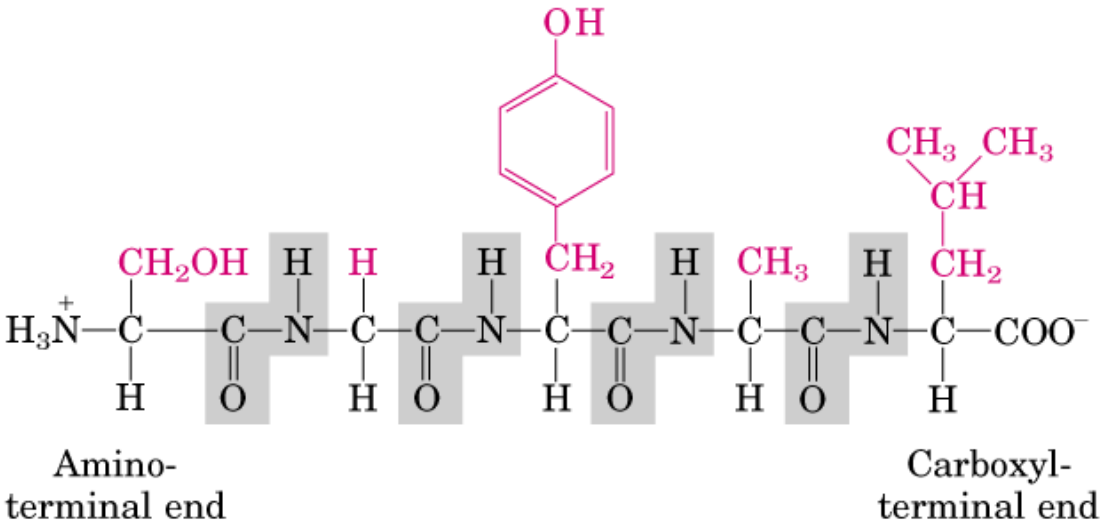


Figure 3: The pentapeptide serylglycyltyrosylalanylleucine, or Ser-Gly-Tyr-Ala-Leu (or SGYAL) showing the N-terminus and C-terminus and the peptide bonds in blue-grey color.[12]

1.2.2 Tryptic digestion

Large proteins can often be cut into peptides by proteolytic enzymes in biological processes and in the study of proteins. Protein identification is often based on the analysis of peptides generated by proteolytic digest. Trypsin is a serine protease found in the digestive system, where it breaks down proteins into smaller peptides or amino acids; it is widely used in biotechnological processes and is commonly used, in proteomics, to digest proteins into peptides for protein analysis [13].

Trypsin specifically hydrolyzes the protein at the carboxyl side of the amino acids lysine and arginine [14], except when either is followed by proline, and this proteolytic cleavage process is known as tryptic digestion. Figure 4 shows a simple picture of the trypsin cleavage in the tryptic digestion.

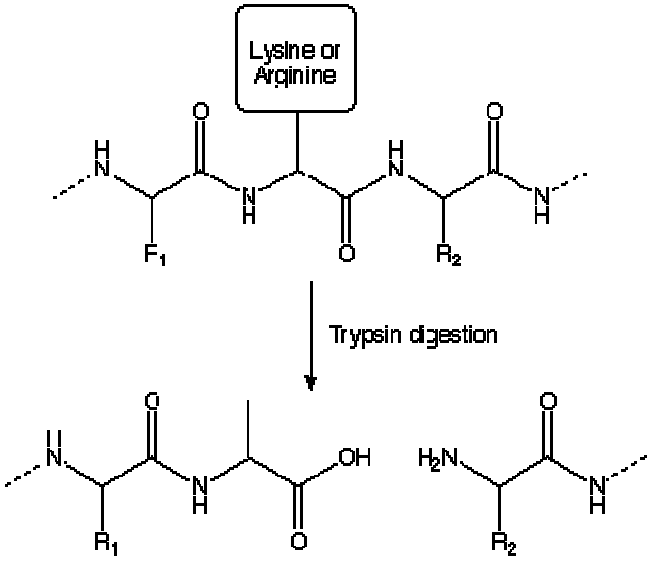


Figure 4: Trypsin cleaves the peptide bond to the right of lysine or arginine residues [15].

The cleaving C-terminal to lysine and arginine residues leads to peptides in the preferred mass range for effective fragmentation by tandem mass spectrometry (MS/MS)[14]. Trypsin has an optimal operating pH of about 8 and optimal operating temperature of about 37°C. However, autolysis (self-cleavage) can also happen when no more other proteins are present in the digestion solution.

1.2.3 Reduction and alkylation

Proteins in fact contain a number of cysteine residues, often linked in pairs to form disulfide bridges. The tertiary structures in proteins are stabilized greatly by disulfide bonds [16], thus breaking these bonds by reduction reaction, unfolds the protein in the way that the efficiency of tryptic digestion increases and improves [17, 18]. Under strongly reductive conditions, the protein disulfide bond can be completely reduced [16].

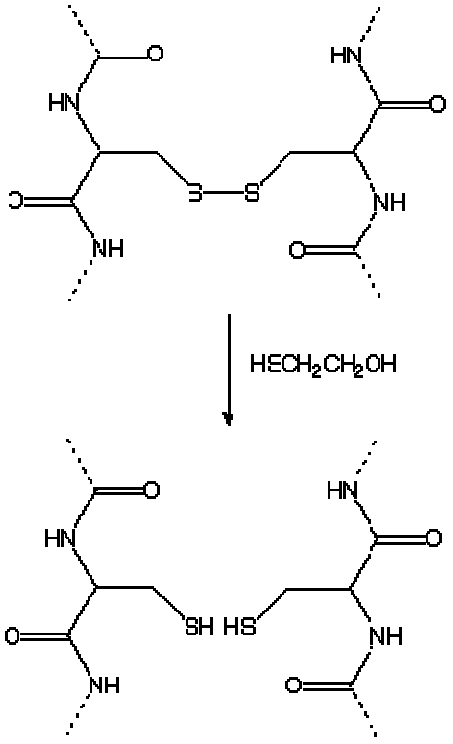


Figure 5: Reduction of the disulfide bond in the protein [15]

Figure 5 shows an example of the reduction reaction done with mercaptoethanol (HS-CH_2-CH_2-OH). Other reduction agent such as DL-dithiothreitol (DTT) is also used quite often in the reduction reaction before tryptic digestion of proteins.

Free sulfhydryl groups are highly reactive and will spontaneously oxidize with other sulfhydryl groups, for this reason these free groups are blocked by alkylation to prevent unwanted reactions from occurring [17]. Figure 6 shows an example of the alkylation reaction with iodoacetic acid, this alkylation reaction can also be done with iodoacetamide (C_2H_4INO) with the same reaction mechanism.

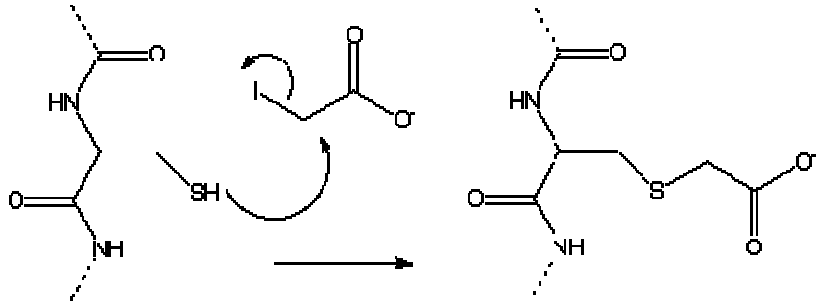


Figure 6: Alkylation reaction mechanism with iodoacetic acid.[15]

In the reduction of protein disulfides must be followed by alkylation and these steps eliminate artifacts in the 2D maps [19]. It is also found necessary to carry out reduction and alkylation of the proteins before tryptic digestion prior to LC and MS analysis [20].

1.3 Protein NMR

Protein nuclear magnetic resonance spectroscopy –Protein NMR is used to obtain information about the structure and dynamics of proteins. Magnetic fields above 500 MHz is generally required for analysis of proteins and peptides [21]. NMR analysis can be done both on unlabeled and ^{13}C, ^{15}N, labelled proteins and analysis of these samples often gives spectra with a large number of peaks. Determination of the nuclei responsible for a given peak involves both determining the amino acid, its sequence position and assignment of which specific nuclei is responsible for a NMR peak. Amide protons are typically found in the one dimensional (1D) and two dimensional (2D) spectrum in the region commonly known as the amide region.

COrrelation SpectroscopY (COSY), TOtal Correlation SpectroscopY (TOCSY) and Nuclear Overhauser Effect SpectroscopY (NOESY) techniques are mostly used in the assignment process and is describes further in chapter 4 of the compendium by Kristiansen [21]. However, in investigation of large polypeptides, Heteronuclear Single - Quantum Correlation (HSQC) experiment is used besides NOESY and TOCSY experiments. With the HSQC experiment, observation (acquisition) is done on protons, and nitrogen (^{15}N) and carbon (^{13}C) nuclei are observed indirectly. The ^{15}N-HSQC is often referred to as fingerprint of protein because each protein has a unique pattern of signal positions. The NH in the protein is seen here. However, it is not possible to assign peaks to specific atoms from HSQC alone. On natural abundance protein the ^{15}N HSQC usually gives the chemical shifts of the hetero atoms as well as help in the assignment of certain amino acid residues such as Gly, Asn, Gln and Trp [21]. Furthermore, ^{1}H-^{15}N HSQC is often used as a technique for investigating the degree of structuring and to improve the solvent conditions of protein sample prior to more time consuming structural NMR work; and in some cases this is used to test whether an unknown sample contains protein for further identification. However, NMR is still a method which usually requires samples in microgram or milligram to obtain spectra that help to elucidate the structure of an unknown sample.

1.4 Capillary Liquid Chromatography

Analysis of proteinaceous binding media in paintings can be done by chromatographic methods [8]. Generally, combination of HPLC with MS has evolved into a sensitive, rugged and widely used technique. Different chromatographic modes such as reverse phase, ion-pair, size- exclusion or immunoaffinity separations are compatible with electrospray (ES) and atmospheric-pressure chemical ionization (APCI) MS [22]. However, ES LC-MS is best done with capillary liquid chromatography (cap LC). Cap LC uses smaller column internal diameters (I.D.) than conventional HPLC. Smaller I.D. columns provide better detection limits for electrospray mass spectrometry, which is concentration sensitive. Furthermore, the sensitivity of electrospray ionization is inversely proportional to flow rate, thus the cap LC system which has low flow rate, is also more favourable for keeping the best sensitivity of this ionization technique.

Cap LC-MS is more and more the method of choice for the separation and identification of complex protein and peptide mixtures [23-26] and even quite complicated system like SPE-HILIC-SPE-RP-MS [*]can be performed online for separation of complex peptide samples [27].

1.5 Mass spectrometry

In the last decade mass spectrometry has emerged as a prominent detection technique for determination of biological molecules [28]. The fast instrumentation improvements have made mass analyzers applicable in the bioresearch [29]. Different MS instruments can be used such as Time-of-flight (TOF), Ion trap (IT), Ion trap-Linear ion trap (IT-LIT), hybrid quadrupole time-of-flight (Q-Q-TOF), tandem time-of-flight (TOF-TOF), Fourier transform-ion cyclotron resonance (FT-ICR), triple quadrupole (Q-Q-Q) and quadrupole ion trap (Q-Q-LIT) [30]. Data collected from the mass spectrometers can be used either to measure simply the mass of a polypeptide or to determine additional structure features. MS-based strategies have become the most commonly used techniques for compound identification in bioresearch.

[*] Solid Phase Extraction - Hydrophilic interaction chromatography - Solid Phase Extraction - Reverse Phase - MS

Electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) coupled with MS give high sensitivity, high specificity and likewise structure information [31]. With the tandem mass spectrometry, MS/MS modes of operation, amino acid sequence of a specific peptide can also be determined [31, 32]. This technique is possible on instruments equipped with MS/MS capabilities.

The high mass resolution of the time-of-flight (TOF) MS has made MALDI-TOF MS a standard tool in proteomic studies and enables precise mass-to-charge (*m/z*) measurement and compound identification [33].

Ion trap mass analyzer is characterized by MS/MS capabilities with unmatched sensitivity and fast data acquisition, though it lacks mass measurement accuracy due to limited-resolution, low-ion trapping capacity and space-charging effects [30]. Their unique capacity to trap certain ions, and subsequently fragment them a few times, results in detailed compound structural information, and this type of instrument has been implemented in many applications. However, the choice of MS platform and favorable strategy depend on whether the application focuses on identification or quantification, (and the availability of mass spectrometers at the place and time of analysis is also a consideration factor in an analysis process).

Mass spectrometry-based proteomic workflow consists of three distinct stages. Firstly, protein samples are isolated from their biological sources and optionally fractionated, the final protein sample is then digested with an enzyme and the resulting peptide sample is further fractionated. Secondly, the peptides are subjected to qualitative and quantitative mass-spectrometric analysis. Finally, the large data sets generated are analyzed by suitable software tools to deduce the amino acid sequence and the quantity of the proteins in sample if applied. The peptide identification is assigned to the MS/MS spectra by database searching.

1.6 Aim of Study

A sample from the Scream painting which had been analyzed by Infrared Spectroscopy (IR) with the diamond attenuated total reflectance (ATR) technique, and found to contain amide bands [34], was to be further characterized by different methods to reveal the type of glue

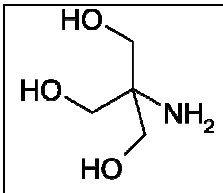
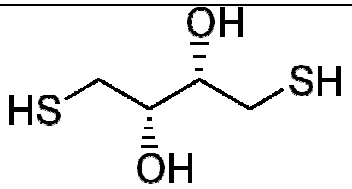
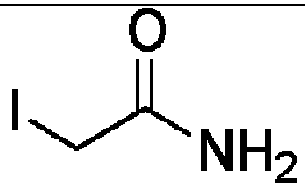
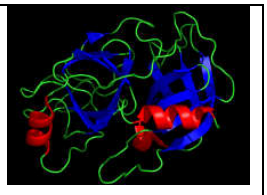
use. With IR, determination of the presence of proteinaceous materials on the basis of the characteristic amide bands is possible, though the identification of a specific binding medium is not generally feasible. The following study aimed at finding analytical procedures, including sample preparation which could provide more information of the glue. A reference glue (from the Munch Museum in Oslo) which was suspected to be similar to the one that Edvard Munch used in the Scream painting was used for the method development, and the Scream painting sample was analyzed by the method developed.

2. EXPERIMENTAL

2.1 Materials and reagents

Deuterium oxide 100 % was obtained from Cambridge Isotope Laboratories, Inc (Andover, MA, USA). Hydrochloric acid (HCl) 37 % solution (analytical grade) was purchased from Merch KGaA (Darmstadt, Germany). Trishydroxymethylaminomethane (Tris or 2-amino-2-hydroxymethyl-1, 3-propanediol), Trizma base reagent grade; DL- Dithiolthreitol (DTT) for electrophoresis 99 %; iodoacetamide (IAM); β-lactoglobulin A (beta-A) from bovine milk; Cytochrome C (cyto-C) from bovine herat and trypsin from bovine pancreas, TPCK[†] treated, were obtained from Sigma Aldrich GmbH (Steinheim, Germany). Acetic acid 50% solution (HPLC grade) was from Fluka (Buchs, Switzerland). Grade 1 water was provided by Milli-Q ultrapure water purification system (Millipore, Bedford, MA, USA). HPLC grade acetonitrile (ACN) was purchased from Rathburn Chemical Ltd. (Walkerburn,UK). HLPC grade water, formic acid (F.a) 50% solution, were obtained from Fluka (Buchs, Switzerland). All fused silica cappilaries were purchased from Polymicro Technologies Inc. (Phoenix, AZ, USA). Nitrogen was produced by a Nitrox N_2-generator from Domnick Hunter (Durham, UK) and helium (99.9999 %) was obtained from AGA (Oslo, Norway). GHP Acrodics syringe filter, 0.45 µm and 0.20 µm, were obtained from Pall Coporation.

Table 1: structure of Tris, DTT, IAM and trypsin molecules

			
Tris molecule	DTT molecule	IAM molecule	Trypsin molecule

[†] Trypsin is treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) to inhibit contaminating chymotrypsin activity without affecting trypsin activity.

2.2 Reference glue samples

The reference glue was obtained from the Munch museum (Olso). The reference glue is a solid consisting of spherical particles in size a bit bigger than a sesame seed. They have brown colour, some are darker brown than the others. One glue bead weighs about 22 mg, the size of these glue beads are quite similar and about 4 mm, though some are a bit bigger or smaller than others. A picture of glue beads is shown in Figure 7.

Figure 7: The reference glue sample in a 7cm plate..

2.3 NMR analysis of reference glue

2.3.1 NMR Sample preparation

131 mg reference glue was dissolved in 900 µL H_2O and 100 µL D_2O, with 30 minutes ultrasonic treatment (Ultrasonic cleaner, USC 100T, from VWR international with an effective power of 30 W). This 131 mg/mL solution was filtrated through a 0.45 µm filter and transferred to a 5mm NMR sample tube (WG-5M, Wilmad glass CO. INC) for NMR analysis.

Another reference glue sample, dissolved in D_2O and H_2O (1:9) and adjusted to pH of 3 with 1 M HCl, was also prepared. pH 3 was obtained by adding 2.5 µL 1 M HCl to a 1 mL

solution of 100 µL D_2O and 900 µL H_2O. To this solution 137.2 mg glue was added and subjected to 5 minutes ultrasonic treatment. The lid of the glass vial was opened to reduce pressure twice dissolving the glue after a total of 15 minutes ultrasonic treatment. This glue solution had pH of 5.5, which was adjusted to pH 3 by adding 70 µL 1 M HCl. The solution was then filtrated through a 0.45 µm filter and submitted to NMR analysis. The pH measurement was done by using pH – paper Acelit pH 0-6 (Merck Darmstadt, Germany).

2.3.2 NMR Experiments

Both 1D and 2D experiments were preformed on the neutral reference glue sample prepared as described in 2.3.1. These NMR experiments were acquired on a 600 MHz NMR instrument, Bruker AV600 with cryo probe (Bruker 5 mm TCI cryo probe ^{1}H, ^{13}C, ^{15}N), with solvent locked on D_2O. The instrument is controlled by three software packages which are installed in parallel on a Dell Windows XP PC: XwinNmr 3.5, patch level 6 (spectrometer license), TopSpin 1.3, patch level 7 (spectrometer license) and TopSpin 2.0, patch level 5 with automatic gradient shimming program Topshim. 1D proton experiment was acquired with pulse program *zgpr*; 1D carbon experiment with pulse program *zgpg30*; 2D HSQC experiments were acquired with pulse program *hsqcetgp* for ^{1}H-^{13}C correlation and *hsqcetf3gp* for ^{1}H-^{15}N correlation. The HSQC experiments were acquired at 311 K with Biotools NMR optimization for H_2O suppression.

In 2D HSQC experiments acquisitions were done with, hsqcetgp and hsqcef3gp, ^{1}H and X-nucleus (^{13}C/ ^{15}N) correlation via double inept transfer, phase sensitive using Echo/Antiecho-TPPI gradient selection, decoupling during acquisition and trim pulses were also used in inept transfer. These acquired spectra were processed and phased to obtain the desired spectra which were used in assignment of the peaks obtained.

The same experiments were also acquired on the reference glue sample with pH 3.

2.4 Total protein measurement of reference glue

2.4.1 Reference glue solutions

A solution of 50 mg/mL reference glue was made by dissolving 204.4 mg glue in 4.08 mL grade 1 water. Other concentration of reference glue solutions were made by diluting this glue solution with water as shown in Table 2.

Table 2: Three glue samples concentrations were prepared with the volume reagents shown.

Glue sample concentration	**Preparation**
10 mg/mL	200 µL (50 mg/mL) glue + 800 µL water
1 mg/mL	100 µL (10 mg/mL) glue + 900µL water
0.3 mg/mL	150 µL (1 mg/mL) glue + 350 µL water

2.4.2 Calibration solutions

Bovine Serum Albumin (BSA) 1 mg/mL solution was obtained from Sigma Aldrich GmbH (Steinheim, Germany). 0.5 mL BSA calibration solutions were made by appropriate dilutions a volume of the 1 mg/mL BSA solution as shown in Table 3.

Table 3: Volume (microliters) of BSA (1 mg/mL) and water used in seven calibration solutions.

Calibration solutions	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Volume 1mg/mL BSA (µL)	0	25	50	75	100	125	150
Volume water (µL)	500	475	450	425	400	375	350

2.4.3 Total protein measurement

The total protein determination of the reference glue was obtained according to the Bradford protein assays with Wallac Victor^3 instrument from Perkin Elmer (model 1420 Multilabel Counter, Wallac Oy, Turku, Finland). 10 microliters of the samples or standards were added to each well in a microliter plate and 200 µL Coomassie reagent was added to all these wells

and incubated at room temperature for 5 minutes. Victor^3 instrument were set to 25º C. The instrument measured each standard and sample twice and the concentrations given are obtained as average from the two series. The method measured total protein concentration between 50 and 350 µg/mL proteins.

2.5 Trypsination procedure

The tryptic cleavage method described on pages 41-42 in Lund's thesis [35] with reduction and alkylation steps was applied. Trypsination procedure in the present study combined the procedure used by Tran [36] with the reagent volumes and concentration from Lund's thesis, and tested with standard proteins and reference glue.

2.5.1 Materials and reagents

DTT, IAM, trypsin, beta-A and cyto-C were the same as listed in 2.1 and structure of DTT, IAM and trypsin is shown in Table 1. A Thermo Orion pH-meter (model 720 Aplus, was calibrated with 2 buffers, BHD buffer solution (phosphate, pH 7.00±0.02) and BDH buffer solution (phthalate, pH 4.00±0.02) before the pH measurement. Both of the BHD buffers were obtained from VWR international Ltd (England).

2.5.2 Solutions and sample preparations

1 M HCl solution was made by diluting 826 µL HCl (37 % solution, 12.1 M) to a volume of 10 mL with grade 1 water.

100 mM tris buffer was made by dissolving 1.21 g Tris firstly in 80 mL water. This solution with pH 10.69 was then adjusted to pH 8.50 with 1 M HCl. The pH-meter was calibrated with 2 buffers before the pH measurement. After approximately 3.5 mL 1 M HCl was added to the tris solution the pH was down to 8.50; this solution was then diluted with water to 100 mL. After the dilution the 100 mM tris buffer (Table 4) was pH checked and the solvent had the pH 8.50.

45 mM DTT solutions was made by firstly weighing out an amount of this reducing agent, secondly, adding the calculated amount of grade 1 water (Table 4), knowing from Lund's

thesis that 6.9 mg DTT is needed to 1mL water. The solutions were made when they were used.

100 mM IAM solution was made by firstly weighing out an amount of IAM, secondly, adding the calculated amount of grade 1 water (Table 4), knowing from Lund's thesis that 56 mg DTT is needed to 3 mL water. This solution was made fresh for the alkylation.

Standard protein solutions, cyto-C and beta-A were prepared by dissolving an amount of standard protein in the appropriate volume of tris buffer (Table 4).

Reference glue solutions, 25 mg/mL glue solution was made by dissolving an amount of glue in tris buffer and ultrasonically treated for 30 minutes. The solution was filtrated with 0.45 µm filter. Other concentrations of the reference glue were made by diluting the reference glue solutions with tris buffer (Table 4)

The 0.01 % (v/v) acetic acid solution had pH 4, was prepared by diluting the acetic acid (50 % solution purchased from Fluka) with Fluka water.

Trypsin solutions, 1 mg/mL trypsin stock solution was prepared by dissolving an amount of tryps in 0.01 % (v/v) acetic acid solution. Other solutions were made by diluting stock solution with 0.01% acetic acid. (Table 4)

Table 4: Overview of the solutions and samples prepared.

100mM Tris buffer	1.21 mg tris + water. pH 8.50 (pH adsjusted with 1M HCl)
Reference glue sample (1 mg/mL)	25 mg/mL: 243.3mg + 9.74 mL tris buffer 10 mg/mL: 400 µL (25 mg/mL) +600 µL tris buffer 1 mg/mL: 100 µL (10 mg/mL) + 900 µL tris buffer
Beta- A (1 mg/mL)	2.4 mg beta-A + 2.4 mL tris buffer
Cyto-C (1 mg/mL)	1.6 mg cyto-C + 1.6 mL tris buffer
45 mM DTT	12.5 mg DTT + 1.81 mL water 14.5 mg DTT + 2.10 mL water
100 mM IAM	18.4 mg IAM + 0.99 mL water
Trypsin **(0.1 mg/mL)** **(0.01 mg/mL)**	1 mg/mL stock solution: 1.8 mg trypsin + 1.8 ml 0.01 acetic acid 0.1 mg/mL: 100 µL (1mg/mL) + 900 µL 0.01 % acetic acid 0.01 mg/mL: 10 µL (1mg/mL) + 990 µL 0.01 % acetic acid

2.5.3 Reduction, alkylation and tryptic digestion

The reduction reaction was conducted in 1.5 mL polypropylene vials by adding DTT to each sample solutions, incubated for 2 hours at 37ºC. After reduction was assumed to be completed and cooling to room temperature, IAM was added and the alkylation reaction was conducted at room temperature, in the dark for 70 minutes. When the alkylation time was ended, excess DTT was added to quench the alkylation and also Tris buffer was added. Finally, trypsin was added and tryptic digestion was carried out at 37º C overnight (16 hours).The trypsination was stopped by freezing the samples at -20ºC and they were kept frozen until use. The samples and reagents in the reactions are listed in Table 5.

Table 5: Overveiw of the reactions and reaction reagents added in microliters unit. Trypsin used for the standard proteins were 0.1 mg/mL trypsin solution. The reference glue sample used 0.01 mg/mL trypsin solution.

Sample solutions	**µL** 1mg/mL	**µLDTT** 45mM	**µL IAM** 100mM	**µL DTT** 45mM	**µL tris buffer**	**µL trypsin**
Beta A (20µg)	20	5	5	20	30	10 (1µg)
Cyto C (20µg)	20	5	5	20	30	10 (1 µg)
Ref. glue (20µg)	20	5	5	20	30	10 (0.1µg)

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2.6 Sample preparation Scream painting

2.6.1 Scream painting sample

The sample from the Scream painting (*S-sample*), with unknown compounds/proteins, consisted of a few tiny pale yellow particles. The sample contained in a 5 mL glass vial was firstly dissolved in 20 µL Tris buffer, using the same pipette tip to work with this solution. The pipette tip was used to repeatedly suck and push out the solution to dissolve the particles. Another 20 µL of Tris buffer was added through the same pipette tip and the sucking and pushing was repeated. Finally 5 µL Tris buffer was added through the same pipette tip before the solution (S-sample) was transferred to a 1.5 mL polypropylene vial and treated as described in 2.6.2.

2.6.2 Reduction, alkylation and tryptic digestion

Three samples, the reference glue sample, the standard protein sample (beta A) and the Scream painting sample, were reduced, alkylated and tryptic digested separately in 1.5 mL polypropylene vials. The reactions were conducted as described in trypsination procedure 2.5.3. The reference glue and beta-A solutions were prepared as described in 2.5.2.

2.7 LC – MS analysis

2.7.1 The LC system

Sample was loaded onto a C_{18} analytical column (0.5 mm I.D. x 150 mm, from Agilent, Germany) with mobile phase (MP) composition 5:95 (v/v) A : B. The loading and gradient mobile phase were delivered with flow rate 10 µL/min, using an Agilent 1100 Series capillary gradient pump (Agilent, Palto Alto, CA, USA) with an incorporated on-line vacuum degasser. The mobile phase reservoir A contained 0.1 % (v/v) F.a. in water and B contained 0.1 % F.a. in ACN. The experimental in the LC systems are listed in Table 6. The outlet of the analytical column was connected directly to the electrospray interface in the MS instrument. The MS instruments were a Micromass LCT TOF-MS (Waters, Manchester,UK) equipped with a Z-spray atmospheric pressure electrospray and a Esquires 3000+ IT mass spectrometer (Bruker Daltons, Bremen, Germany) equipped with a micro flow ESI source.

Table 6: Overview of the LC system and the MS

samples	Injection loop µL	Load time (minutes)	MP Gradient	MS instument
Trysination procedure (2.5)	20	4	5 % -70% B over 60 min.	ESI-TOF/ ESI-IT
Standard protein (beta A)	20	4	5 % -70% B over 60 min.	ESI-IT
Blank gradient (Fluka water)	20	6	5 % - 40 % B over 60min 40%- 70 % B over 15min	ESI-IT
Reference glue	20	6	5 % - 40 % B over 60min 40% - 70% B over 15min	ESI-IT
Scream-sample	20 and 51	6	5 % - 40 % B over 60min 40% - 70% B over 15min	ESI-IT

2.7.2 The MS system

The ESI-IT was operated in positive ion mode with an accumulation time of 200ms. Data acquisition was in the *m/z* range 215-3000, in both MS and Auto MS/MS mode, using Esquire Control 3.5 from Bruker Daltonics. Three precursor ions at a time were used with the MS/MS mode in all the experiments, and the fragmentation amplitude was ramped from 30 to 200 %. The signal intensity threshold for Auto MS/MS mode was set to 5×10^5 counts avoiding unwanted background fragmentation. The desolvation gas (dry gas) flow was set to 6 L/min and drying temperature at 250º C.

2.8 Database search

2.8.1 Data analysis

The data acquired with mass spectrometer were analyzed and processed using Data Analysis 3.1 and Biotools 2.2 software from Bruker Daltonics. The Mascot searching engine (Matrix Science, http://www.matrixscience.com) was used for comparation of MS/MS spectra with the protein sequence database at the National Center for Biotechnology Information (NCBI)

and Mass Spectrometry protein sequence DataBase (MSDB). The chosen enzyme in the search was trypsin, variation of the taxonomy was chosen in the searching of the reference glue and the S-sample. Fixed modification used was carbamidomethyl (C). Variable modifications used were oxidation (M); cleavage by trypsin: cuts C-term side of KR unless next residue is P. Other parameters and procedure for the searching process are described in 8.1.

3. RESULTS AND DISCUSSION

3.1 IR analysis and solubility

The reference glue were provided by the Munch Museum in Oslo for analysis because was suspected to be the glue that Edvard Munch used in the Scream painting which needed restoration as mentioned in 1.1. Some materials from the Scream painting were also provided by the Munch Museum. A Scream painting sample and the reference glue were analyzed by infrared spectroscopy [34].

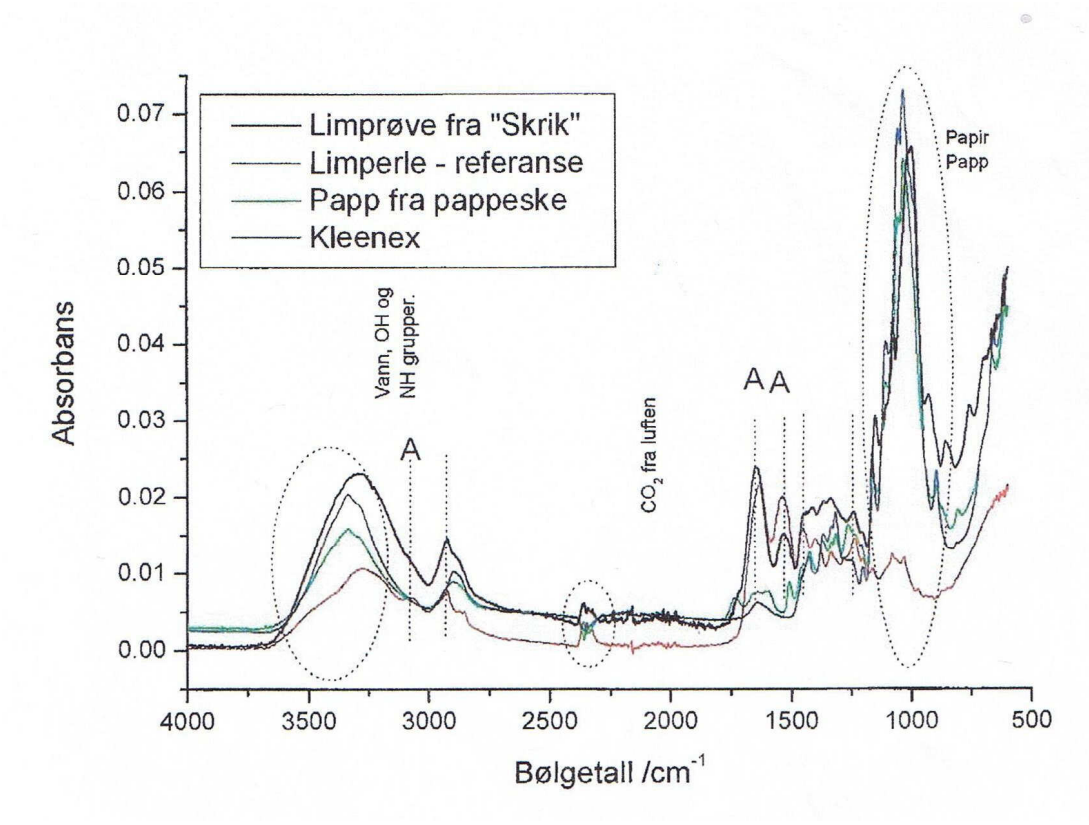


Figure 8: IR spectrum of glue sample (Limperle-referanse) and sample from the Scream (Limprøve fra "Skrik") [34]

The IR spectra obtained show two characteristic amides vibrational bands in both the reference glue and Scream painting. These bands are known as amide-I and amide-II bands due to the secondary amide structure and are noted with A in the spectrum in Figure 8. Further information on the content of the glue was not possible with IR spectroscopy.

However, the amide bands identified in these samples gave ideas for further study and analysis.

To check the solubility, a reference glue bead, 22 mg, was dissolved in 1 mL water with ultra sonic treatment in 10 minutes (i). Four glue beads, totally 96.2 mg, in 1 mL water needed 25 minutes to be dissolved with ultrasonic treatment (ii). After dissolution, the glue solution was pale yellow-brown. Glue solution *i* could be filtrated through a 0.45 µm filter, but did go through a 0.20 µm filter. The second glue solution was more concentrated and was difficult to filtrate. When the glue solution was left at room temperature over night, the solution became solid, but got back to liquid like when warmed up a few minutes in warm water. Dissolving the sample in an appropriate solvent is necessary for many analytical techniques.

When the reference glue was dissolved in solution, to remove particles and components that might interfere with the analysis, a filter was used.

The primary function of most filters is to remove or reduce bacteria and fungi that can contaminate and interfere with protein analysis, as well as other contaminations. For this purpose filters with pore size 0.20 µm is preferred. Since the glue solution was sticky and thick, the 0.2 µm could not be used. And if the concentration of the reference glue needed was less than 22 mg/mL, the sample could be diluted so that it can go though the 0.2 µm filter; but this was not the case when sample was prepared for NMR analysis and other purposes. Another consideration is the fact that the protein concentration in glue might be so low that the concentration in diluted glue is too low for analysis. Therefore, 0.45 µm filter was chosen in further glue sample preparations as described in the experimental

3.2 Protein NMR

One of the important parts of the NMR instrument is the magnet, and as mentioned earlier in 1.3, the magnet field needs to be at least 500 MHz for protein analysis. Another important part is the probe which is responsible for holding the sample, transmitting magnetic pulses and observing the signals. Different probes are designed for different applications, thus it is important to have the proper probe when performing NMR on biomolecules such as proteins and peptides. The cryoprobe was designed for one of these purposes and was used in the

NMR experiments in this study as described in 2.3.2. Generally, in the protein structure determination process there are 4 basic steps that must be performed to obtain structure information. These steps are described in chapter 4 of the compendium by Kristiansen [21], and will not be discussed here. However, data obtained by the HSQC experiments in this study are of great interest. The HSQC with ^{1}H -^{13}C correlation, when well resolved, may assign most of the amino acid sequences since the ^{13}C chemical shift is more amino acid specific than the proton shift values.

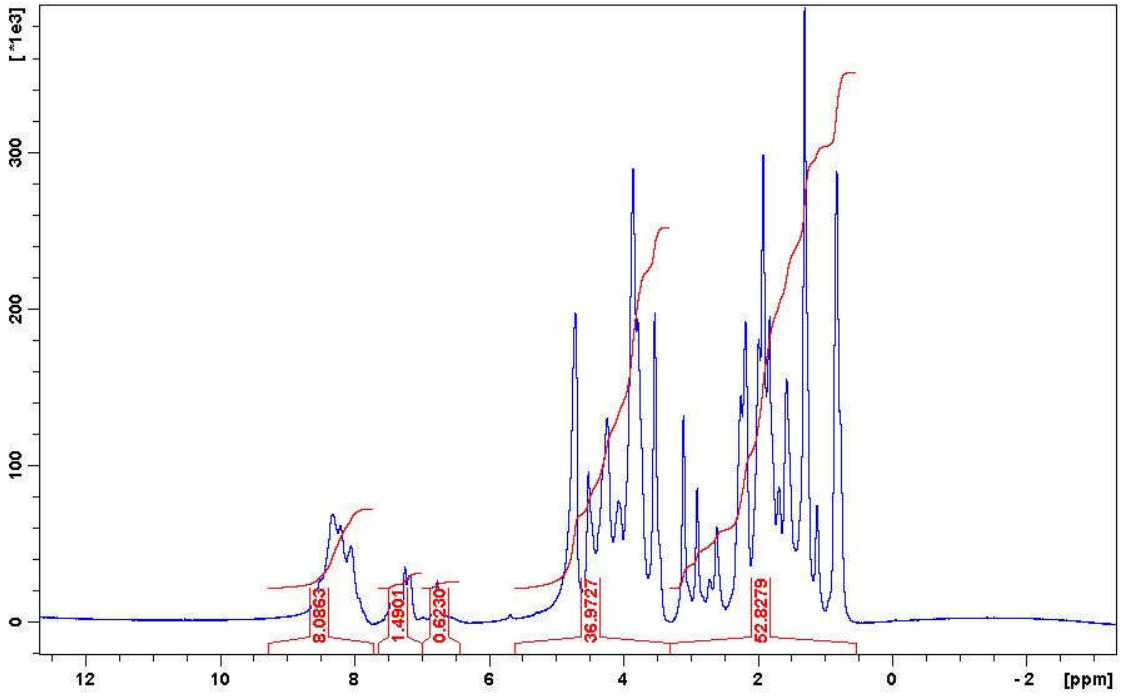


Figure 9: Proton spectrum of the reference glue with integrated values.

The proton spectrum in Figure 9 shows that protons in the glue sample have aliphatic, aromatic and amide shift. Protons belonging to amino groups in peptides or proteins are usually in the shift region 7-10 ppm. Based on the proton spectrum and the carbon spectrum (Figure 10), information from these 1D experiments alone give as expected not enough information to characterize and identify the content of the reference glue, however, they are the initial experiments for the HSQC experiments which will be more discussed below.

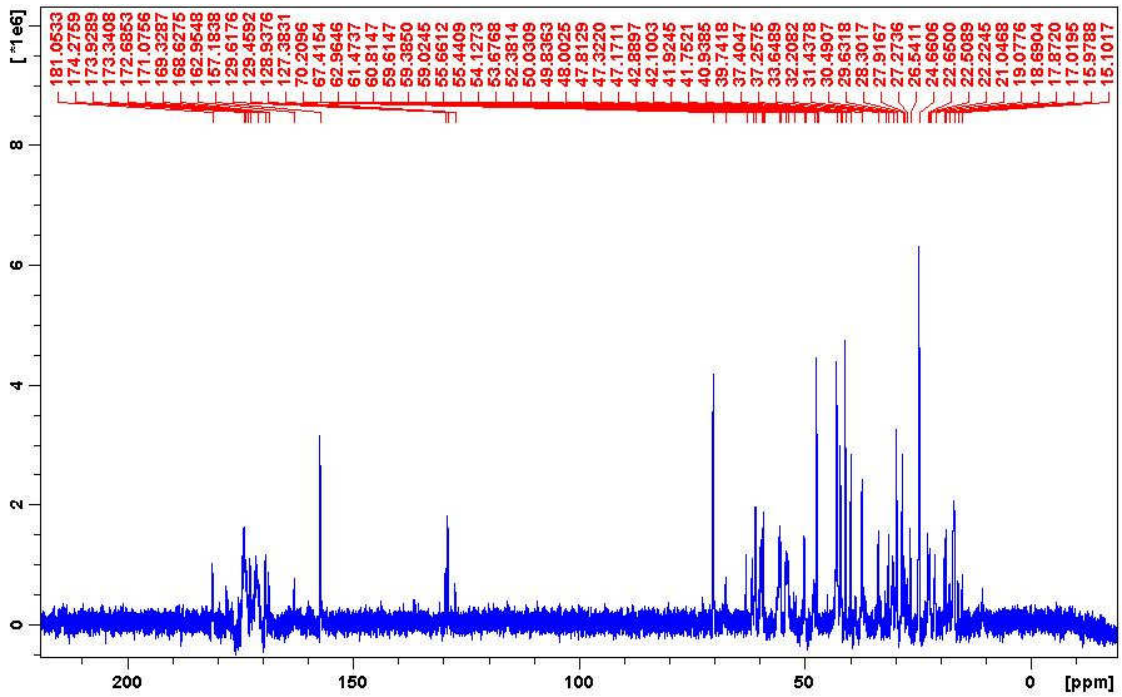


Figure 10: Carbon spectrum of the reference glue with peak labels

The ^{1}H - ^{13}C HSQC spectrum in Figure 11 shows correlated peaks and peak patterns that have characteristics usually found in proteins and peptides. Aromat-CH are in the region 6-8 ppm (F2) and side chain CH-CH_2-CH are in the region 1-5 ppm (F2) (Figure 11). The big green line in the middle of the spectrum is not included as peaks since this is the strong water signal line from the solvent.

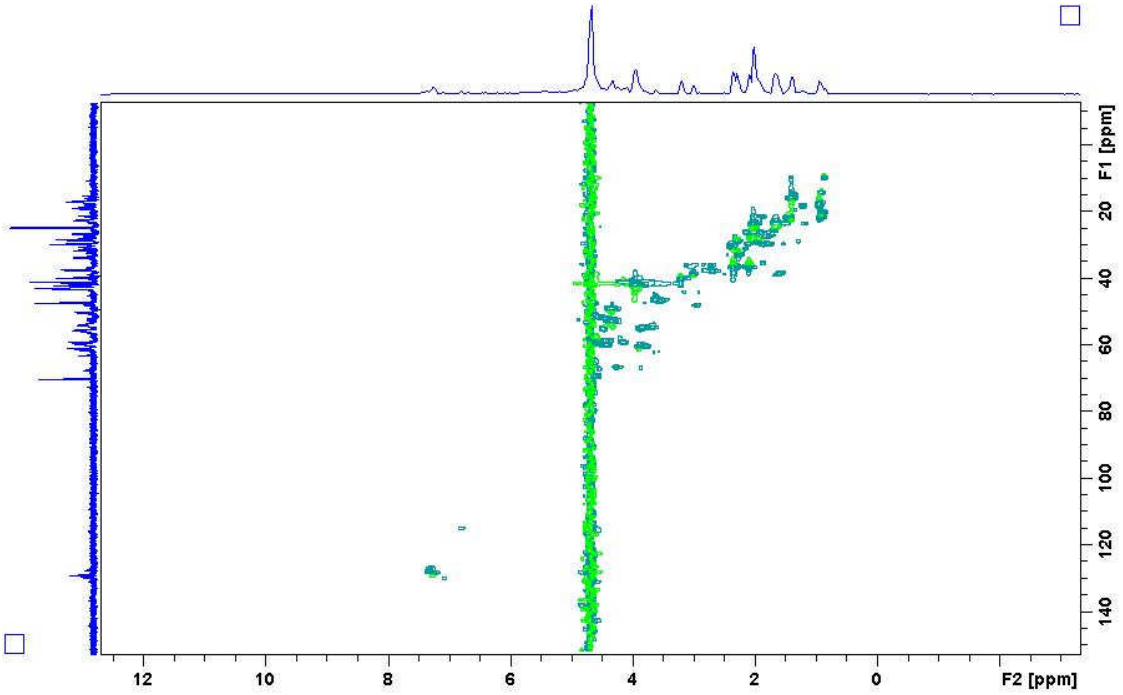


Figure 11: NMR spectrum of the 1H - 13C HSQC of the reference glue, with the carbon on the left spectrum, the F1 axis; and proton spectrum on the top, F2 axis.

Furthermore, a closer look on the ^{1}H-^{15}N HSQC spectrum in Figure 12, identifies the amino acid glycine (G) at 108 ppm on the N –axis and the duplet between 112 and 113 ppm belongs to asparagines (N) and glutamine (Q). This confirmed the fact that ^{15}N HSQC give not only the chemical shifts of the hetero atoms, but also help in the assignment of certain amino acid residues such as Gly, Asn and Gln which were found here. The assignments of these amino acid residues are circled as shown in Figure 12. Each residue of the protein (except proline) has an amide proton attached to a nitrogen atom in the peptide bond. The number of peaks in the spectrum should match the number of residues in the protein (though side chains with nitrogen bonded protons will add additional peaks). However, it is not possible to particularly identify the proteins in the glue due to the fact that these spectra are average spectra of many proteins; only signals of the amino acids in the amide bonds that are in plural are seen here.

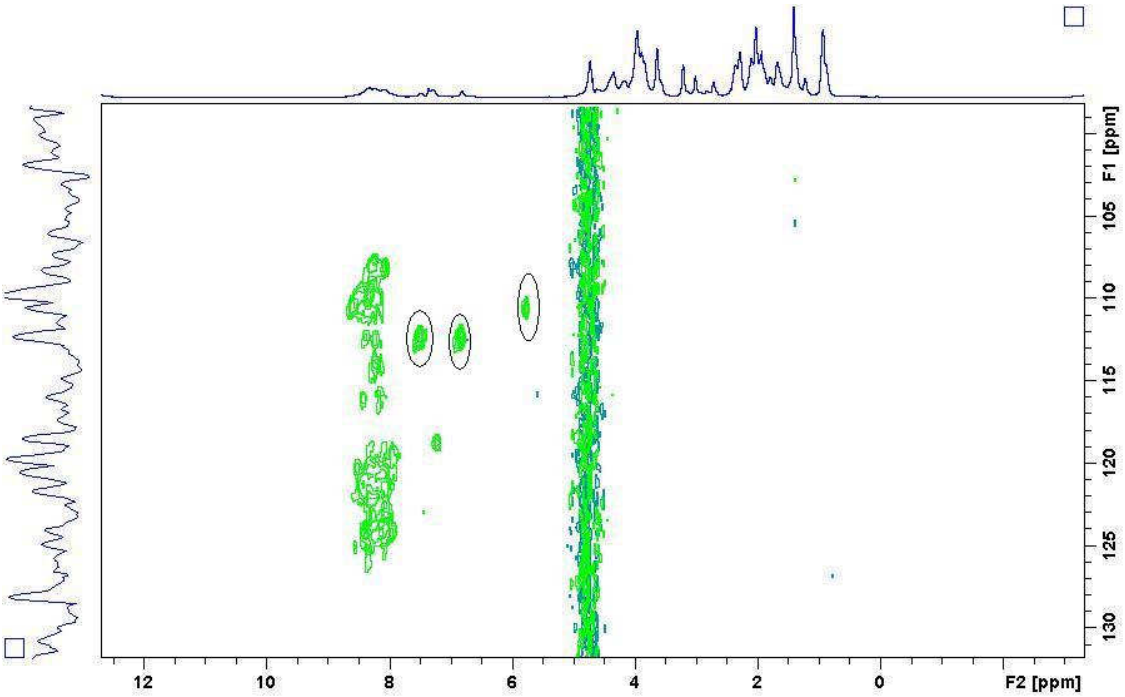


Figure 12: 1H -15N HSQC spectrum of the reference glue with 15N spectrum on the left side, F1 axis; and the proton spectrum on the top, F2 axis.

Though not able to identify what kind of proteins that are presented here, these spectra confirm that the reference glue contains proteins, as found in the IR analysis [34] The amount sample from the Scream painting was too small and could not be analyzed by NMR.

A sample of the reference glue was pH adjusted to 3 since this often gives more signal information in protein NMR analysis. However, the experiments done on this sample did not give better results than these represented above. This is though quite reasonable since the glue has been made by boiling processes and the proteins in the sample might have been degraded or affected in these processes.

3.3 Total protein measurement of reference glue

During testing of this trypsination method for the reference glue sample, it was found necessary to know the amount of protein in the glue sample. This is due to the fact, as mentioned in 1.2.2, that trypsin autolysis and the amount of protein is of important for efficient trypsination. Autolysis of trypsin is not wanted since its peptides will interfere with identification of peptides from the target sample. In trypsination procedures, trypsin was

found to be among the highest score proteins in the Mascot search of the tryptic digested glue sample.

The total amount of protein in the reference glue was determinated by the Bradford Assay [37, 38]. This method is based on the direct binding of Coomassie brilliant blue G-250 dye (CBBG) to proteins at arginine, tryptophan, tyrosine, histidine and phenyalanine residues. Anionic CBBG binds to these residues producing an absorbance maximum at 595 nm; thus the CBBG complex with proteins is measured at 595 nm in a spectrophotometer. The free dye in solution has an absorbance at 470 nm [39]. The concentration of the target protein is calculated by the instrument using a calibation curve made by a standard protein with known concentrations. The total protein measurement results are shown in Table 7.

Table 7. The amount and procents of the protein in the reference glue obtained protein by Bradford method.

Reference glue samples concentrations	**Protein concentrations obtained**	**Procent of the protein in the glue samples**
300 µg/mL	23.2 µg/mL	7.73
1 mg/mL	78.2 µg/mL	7.82
10 mg/mL	264.1 µg/mL	2.64

The concentration obtained for the third glue sample is quite different from the first two as listed (Table 7). The protein measurement method functions in certain range of protein concentration [37, 40] Another fact is that the 3rd sample was much more concentrated and the Coomassie reagents might not be able to complex with this sample so well to get good absorbance for the measurement. Hence, the results from the first two concentrations are more reliable with this method.

Information which was obtained by the total protein measurement listed above the amino acids that are related to this assay. The primary response to arginine is eight times as much as other listed amino acid residues, and there are variation of the others responses too, dependent on the protein, therefore the calibration protein should contain the complexing residues as similar as possible to the analyte proteins. However this is difficult to match in our case since the protein sample is yet unknown. However, in the present case it was not the purpose of the investigation to get the exact protein concentration, hence bovine serum

albumin (BSA) was chosen as calibration protein since it is often used with good result with this assay. The reference glue sample showed less than 10 % (w/w) total protein concentration. Hence in the further experiments, a trypsin amount corresponding to this protein content was used as in 2.5.3

3.4 Tryptic digestion procedure

3.4.1 Tryptic digestion of standard proteins and reference glue

The trypsination was tested using the procedure described in Lund's thesis with reduction and alkylation steps, but in our hands the digested samples showed no or few peptides peaks (e.g. double charged) when analyzed in LC-MS (ESI-TOF). The volume of TEA buffer (480 μL) added before trypsination were considered to be too large for the small painting sample. An alterative procedure used by Tran [36] combined with volumes and concentrations used in Lund' thesis, was tested. The combination of the two procedures in Lund's thesis [35] and Tran's article [36] were based on a close study and calculation of the amount reagents used in the two procedures. On calculation of the mol amount of DTT and IAM which were used by Lund and Tran, the amount reagents are the same according to the amount protein which submitted to be reducted and alkylated. The protein amount (1mg) used by Tran is 50 times more than Lund's. Thus the new procedure as described in 2.5.3 combined the two procedures by keeping the small reagent volume and concentration in the procedure in Lund's thesis and using Tran's article reaction steps. This reduction, alkylation and trypsination method was tested for the reference glue and standard proteins. And the digested samples were analyzed by LC-MS.

The ratio of amount trypsin: protein can be from 1: 10 to 1:50, in the present study a 1:20 protease ratio was chosen, taking into account the unknown protein compositions and knowing that if there are more than 10% protein in the sample there are still enough trypsin. In case of less protein, the amount of trypsin is not too high. Another fact that was found by measuring the protein concentration is that the glue also contains other matter than proteinaceous material. However, since the assay showed the presence of amino acids, most

probably also arginine, trypsinination with subsequent peptide identification, could be applied for protein identification.

The reference glue and standard proteins which were tryptic digested by this procedure gave lots of peptide peaks and the protein identification search (Mascot search engine) showed high scores for the β-lactoglobulin-A. The tryptic digested samples analyzed by LC-MS method showed separated peptide peaks (Figure 13).

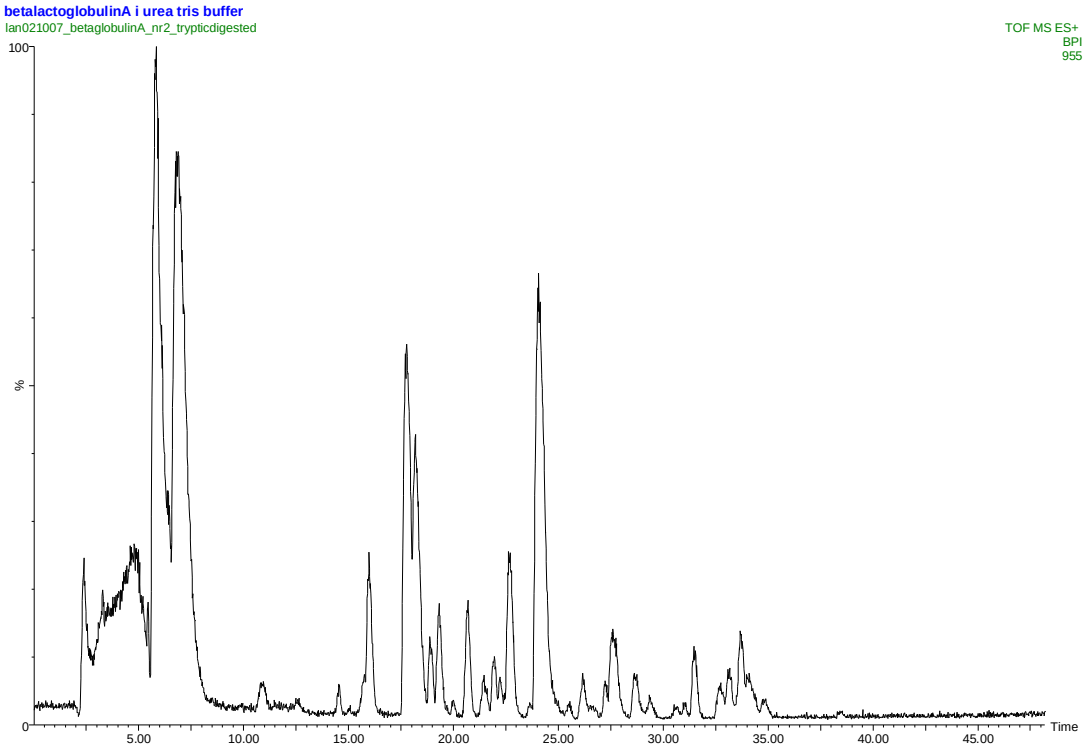


Figure 13: Chromatogram of the Beta A analyzed by LC-ESI-TOF-MS

When a scan over the chromatogram from 16-37 minutes, a lots of peptide masses revealed in the mass spectrum (Figure 14). Figure 15 shows the mass spectrum of a doubly charged peptide.

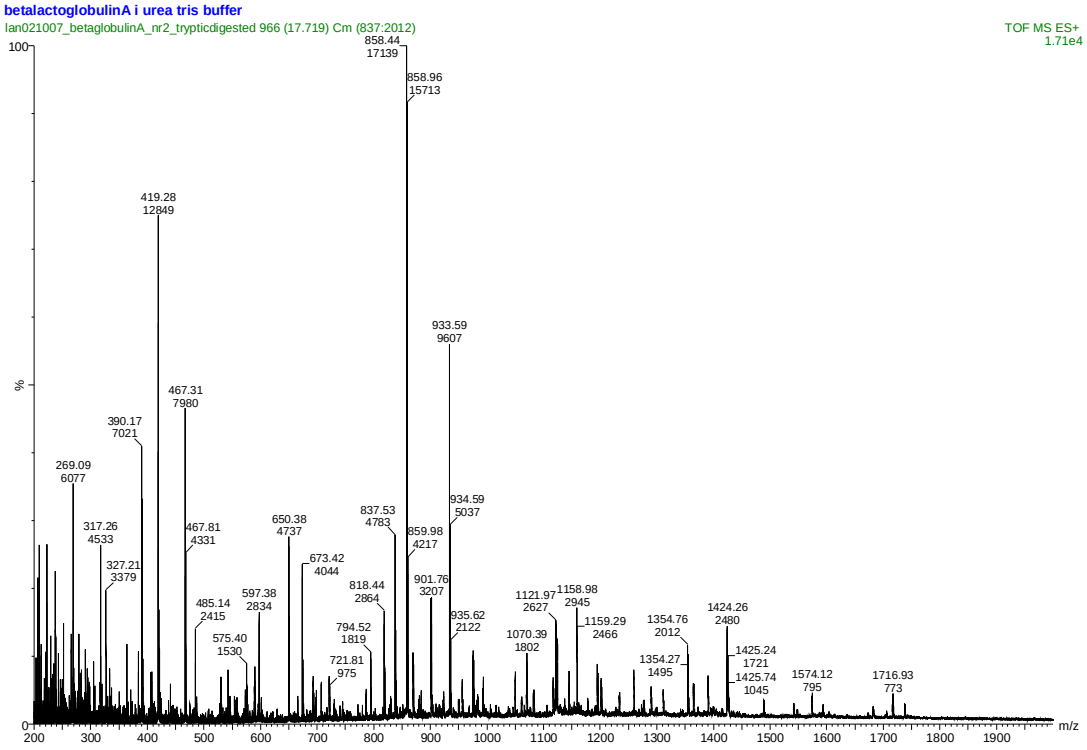


Figure 14: Mass spectrum of the tryptic digested beta-A, scanned from 16-37 minutes in the cromatogram.

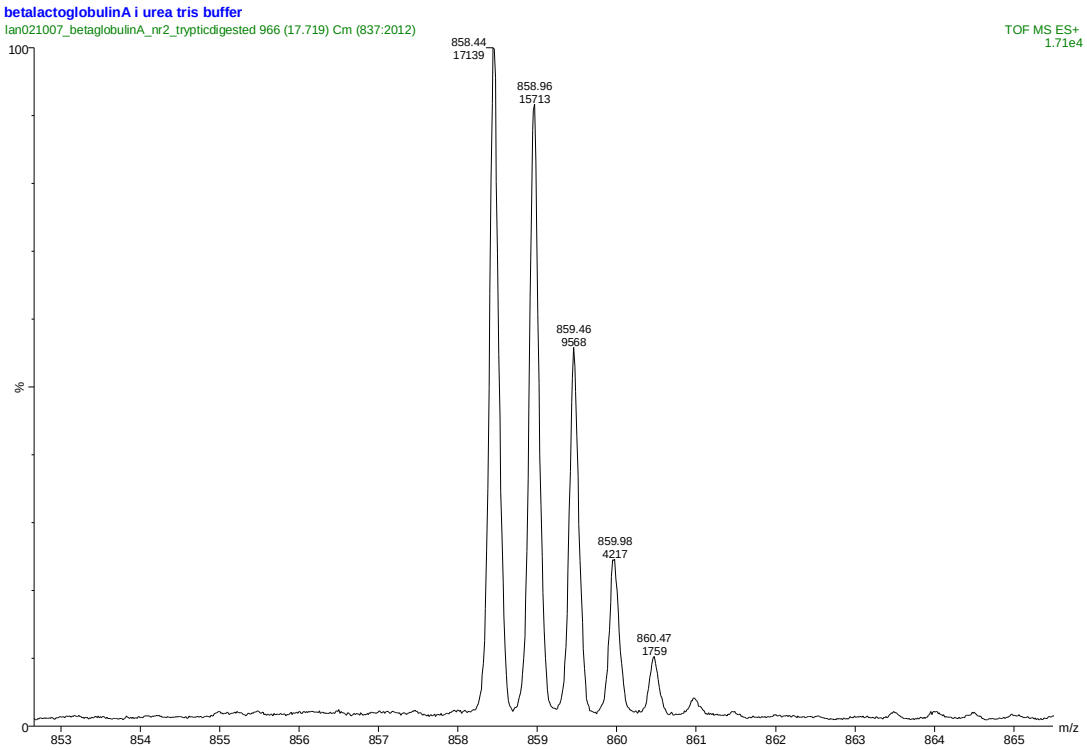


Figure 15: Mass spectrum of the tryptic digested beta-A..This spectrum showed a zoomed-in doubly charged peptide peak with m/z 859.44, 858.96, 859.46, 859.96.

Tris buffer and Urea tris buffer were used in the testing of trypsination procedure. Urea was used to unfold proteins for better tryptic digestion. But, the results from the Urea tris buffer trypsination of the reference glue showed few peptide peaks. Thus tris buffer was used in the trypsination procedure. The proteins in the reference glue might be unfolded or degraded due to the boiling process.

The *m/z* values obtained as doubly and triply charged by the TOF-MS instrument needed to be converted to single mass values before submitting to the Mascot search engine for peptide fingerprint protein identification. For the whole chromatogram one peptide peak at a time needed to be looked up to get all the masses of interest. The singly charged masses obtained were submitted to Mascot. One of the Mascot search results showed high score (158) of the Beta A protein with 100 % sequence coverage when both calculated doubly and triply charged mass were submitted.. This confirmed that the tryptic digestion procedure worked. Mascot search results for the standard protein are shown in 8.2.

The reference glue was also analyzed in the same way and peptide peaks were also found here which confirmed the trypsination of the sample. A chromatogram and mass spectrum of the digested reference glue is shown in Figure 16 and Figure 17. Both doubly and triply charged masses were found when the mass spectra of the glue sample were studied. The chromatogram showed more than 30 peaks in the glue sample that can be peptides and these peaks each revealed many masses.

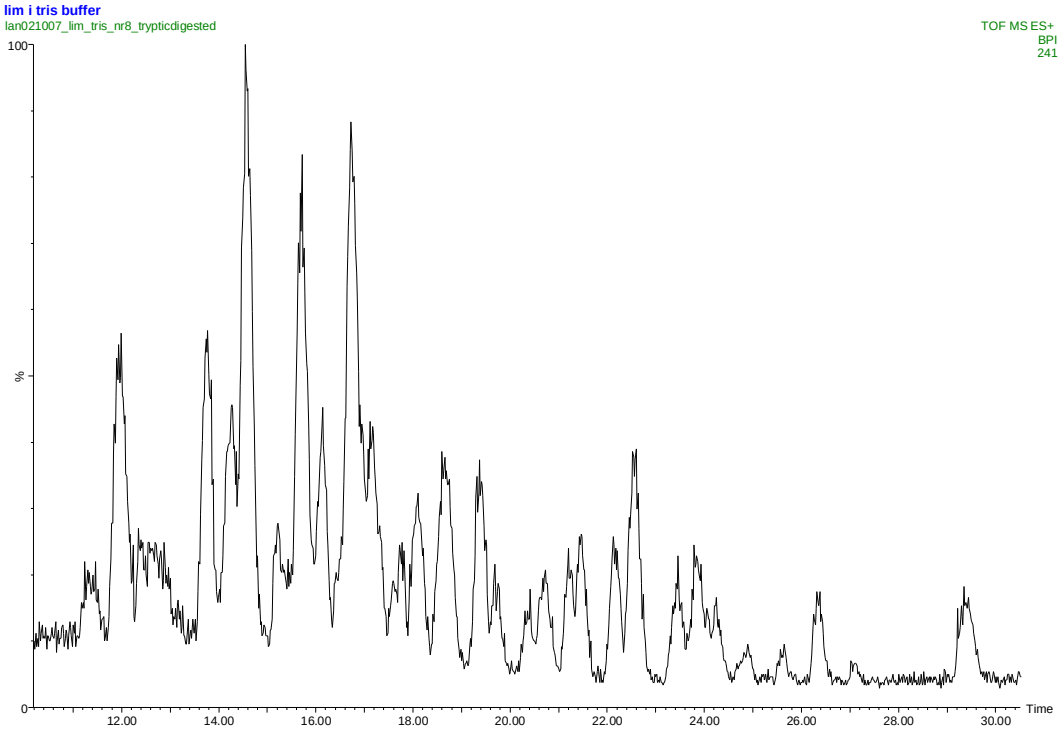


Figure 16: Chromatogram of the tryptic digested reference glue analyzed by LC-ESI-TOF-MS

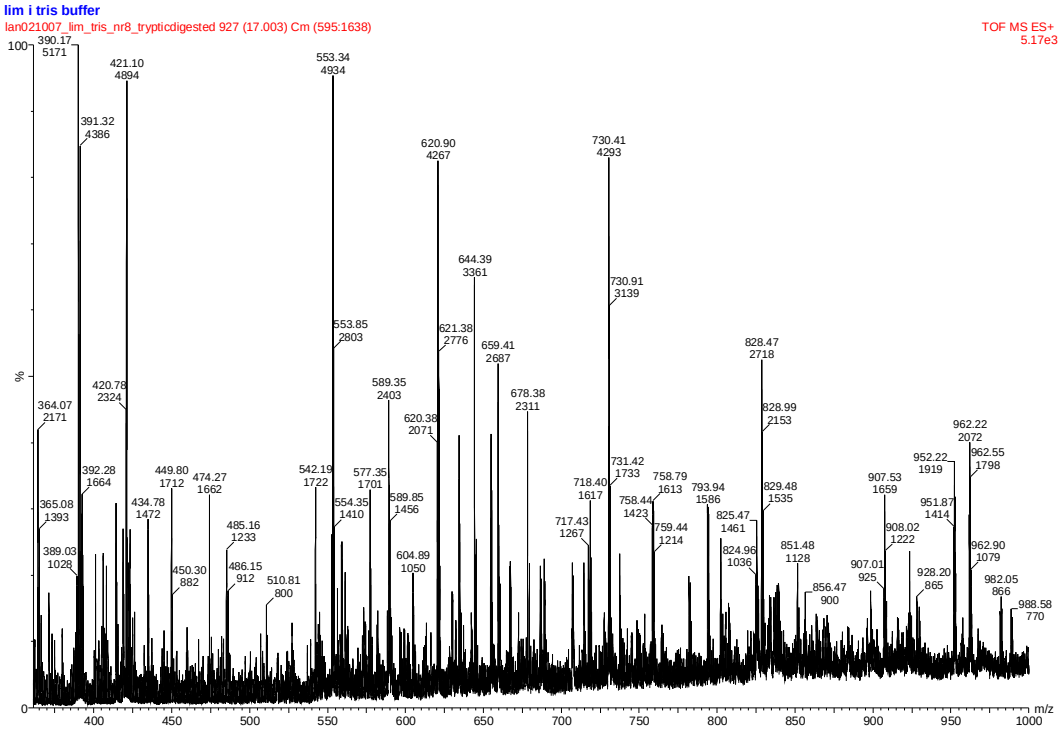


Figure 17: Mass spectrum of the tryptic digested reference glue analyzed by LC-ESI-TOF-MS. This spectrum is a zoomed-in spectrum of the mass spectrum scanned from 12-30 min of the chromatogram above.

The trypsination procedure was also tested with small sample volumes as described in 2.5.3. The ion trap mass spectrometer was used for detection later on since it can provide tandem MS/MS. The chromatograms from the procedure trypsination as described 2.5.3 are shown in Figure 18 and Figure 19. The digested sample showed peptide peaks well separated for the standard protein beta-A, though the reference glue sample might need other separation time and gradient steps for better separation.

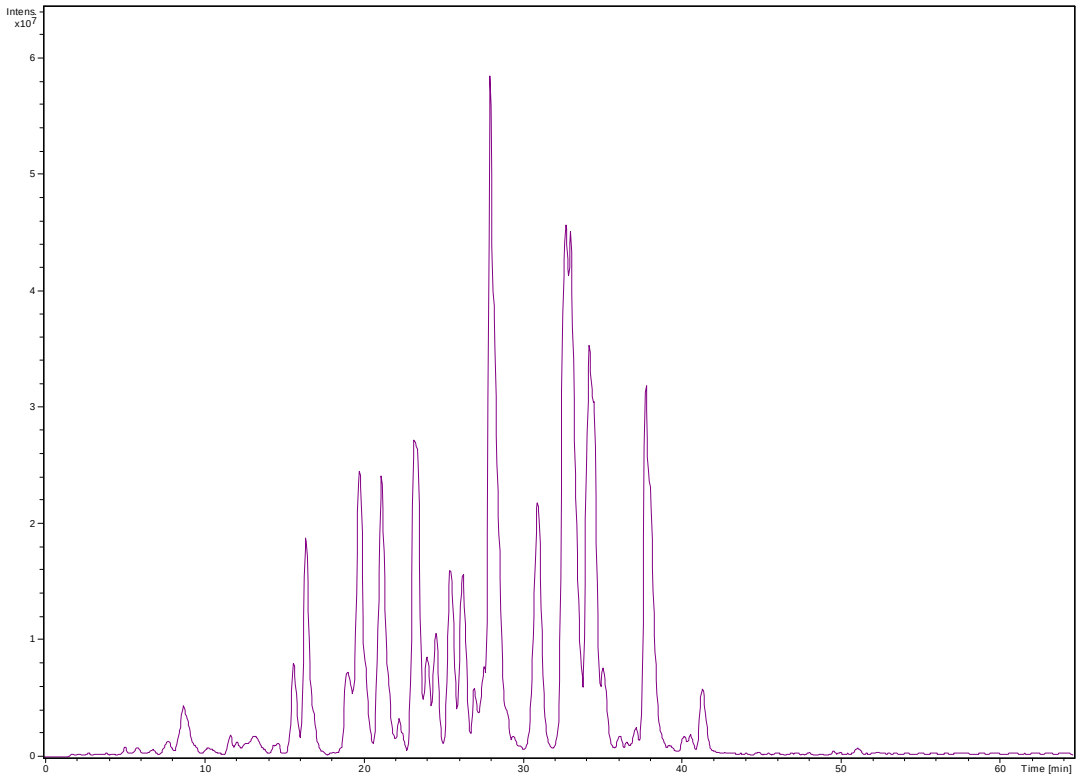


Figure 18: chromtogram of the tryptic digested beta-A (20 µg).

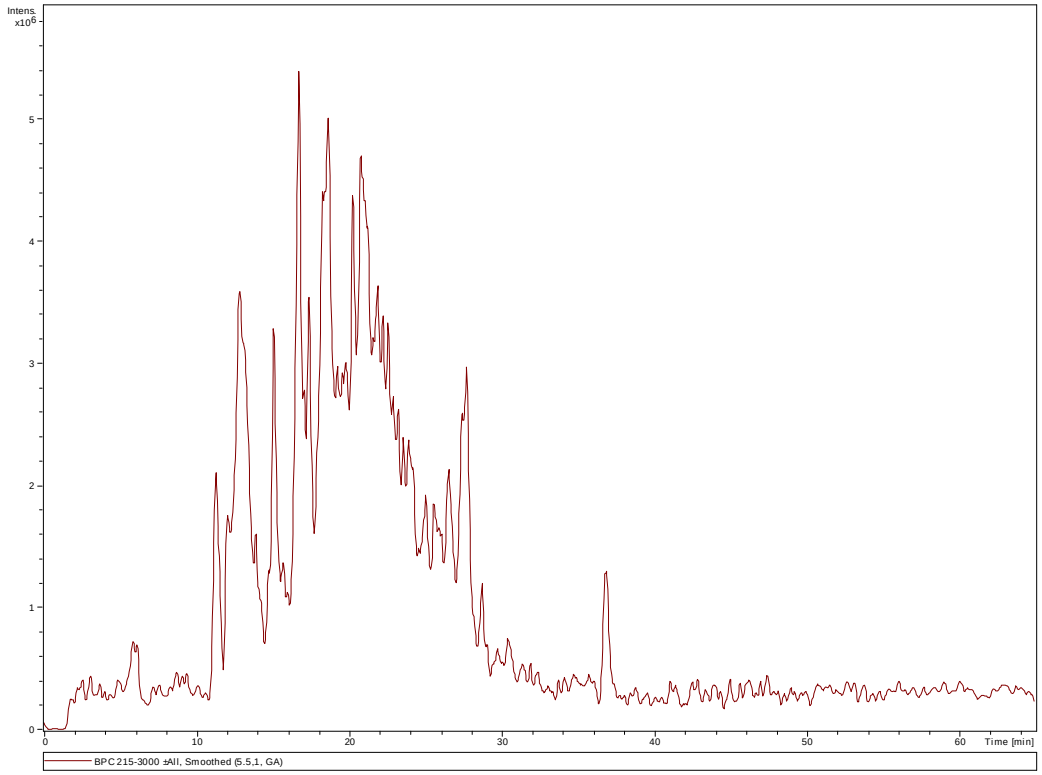


Figure 19: Chromatogram of the reference glue. A sample of 20 µg was tryptic digested.

When MS data of this tryptic digested beta-A sample were submitted to Mascot search engine, the protein identification score for this protein is 459 and with 68 % sequence coverage.This again confirmed that the trypsination method was well functioning.When the reference glue sample was submitted to Mascot search, no significant protein was identified. However, peptides were found, (shown in red in the search results) and some of them are listed in Table 8.

Table 8: some peptides were found in the mascot search of the glue sample.

MLNASVSASTANDGEPILHSEVGR.Q + Oxidation (M) (observed mass 824.430)
FTSMLLIGEAAGYGSR.L + Oxidation (M) (563.16)
FSINFFTAIGLGALTSSMR.K (678.59)
LMNQAGVSFAILGNTEANSGDTAR.R + Oxidation (M) (818.14)
MTTLCYALGIELVPLAGATSCGAGIVR.Q (931.84)
AANTANIPIINAGDGPGQHPTQALLDMYTIK.R + Oxidation (M) (1074.44)
QPFVALSGPSFALELMNNLPTAMVVASK.D + 2 Oxidation (M) (989.34)
LPNSDMTAGNLALIGPASMMNIAMK.A + 3 Oxidation (M) (869.85)
AMPYSDLFLMAIGLAVSAIPEGLPVAISVALAIGMR.R (1219.80)
AWHNPTTNELIAAAAGGIVVVGGIAVLVLITVLR.R (1137.16)
GTMHLGDDAVAAIESHR.W + Oxidation (M) (897.84)
GDIGPAGLPGPR.G (553.36)

Peptides from trypsin were not found listed in the top of the search results. Collagen from rabbit and mouse were suggested to match the peptide (553.36), though the score obtained was not significant for identification. The peptides were not submitted to further identification or peptide confirmation. They are listed as found in the search. The sample from the Scream painting was to be prepared and analyzed with this method.

3.5 LC-MS analysis of Sream painting

Trypsination of the dissolved Scream painting sample (S-sample) was carried out using the trypsination method as described in 2.5 and 3.4.

3.5.1 LC separations

Using a blank gradient before injection of the sample gives control and verification for the peaks of the sample in the system. With 20µL injection of the S-sample showed peptide peaks which were doubly charged, but the peaks were very small. Therefore the rest of the sample volume, only 47 µL, was injected with the bigger loop which was prepared for this step. The data obtained for the S- sample with the second loop was subjected to data analysis.

The position of a peak in a chromatogram is characterized by its retention time (t_R). The chromatogram of the S-sample and the chromatogram of the reference glue show different peak patterns. It was of great interest to figure out whether the reference glue examined or a similar one was used in the Scream painting. To leave out other factors that can unnecessary contribute to changes in the chromatograms, these two samples' introduction and separation conditions were kept alike. Hence, if the two chromatograms show similar peaks and peak patterns, they might contain the same components and materials. However, by comparing the peaks and their pattern in the chromatograms of the reference glue sample and the S- sample in overlaid chromatograms as showed in Figure 20 it can seen that the blue and black chromatograms do not match each other in all peaks and patterns. Hence, the glues were not identical.

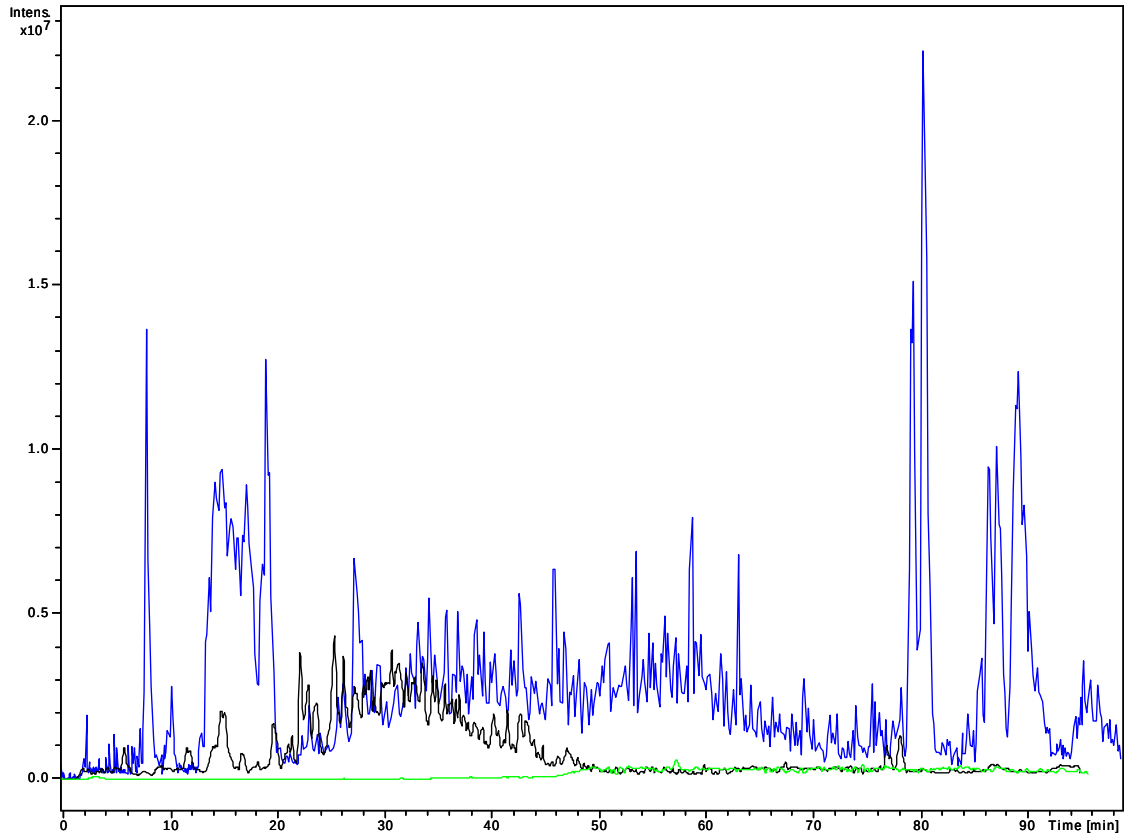


Figure 20: The chromatogram of the S-sample is in blue colour, the black is the reference glue and the green is the blank gradient chromatogram.

Another fact is that the S-sample is a mixture of glue, colour pigments and probably cardboard materials, and was aged with the painting, while the glue sample is a mixture of animal tissues making it the glue. In a LC system, it is not possible to extract one peak from other peaks in the chromatogram. However, in a LC-MS system, it is possible to get an extracted ion chromatogram when a mass of interest is applied. This will be discussed later in 3.5.2. At this point the reference glue does not seem to be the same one used in the Scream. Further identification needs to be done and analysis by mass spectrometry to see whether it would bring this matter closer.

3.5.2 Mass spectrometry

Automated tandem mass spectrometry or MS/MS data-dependent acquisition mode is the most commonly used technique for detection and identifying peptides/proteins, and amino acid sequence of a specific peptide can also be determined. As each peptide elutes from the

chromatographic system, the instrument automatically select collision induced dissociation (CID) of the parent/precursor ions using automated scan functions. Target MS analyses use the optimal tune parameters to obtain the best performance for the target compound. However, with this data dependent acquisition (DDA) mode, it is necessary to use average parameter settings in order to detect as many compounds as possible in a sample. To ensure complete compound information three precursor ions at a time were used with the MS/MS mode in all the experiments studied using the ion trap instrument. When a mass in a compound were detected as a precursor ion, the ion would be further fragmented and a tandem MS/MS mass spectrum obtained. An example of a MS spectrum and a MS/MS spectrum shown in Figure 21and Figure 22.The red mark (Figure 21) indicated that the *m/z* 423.1 was fragmented further. The MS/MS spectrum obtained (Figure 22), was not a peptide, since it revealed few masses and all below the blue mark. An example of a peptide MS/MS spectrum will be shown later in 3.5.3.

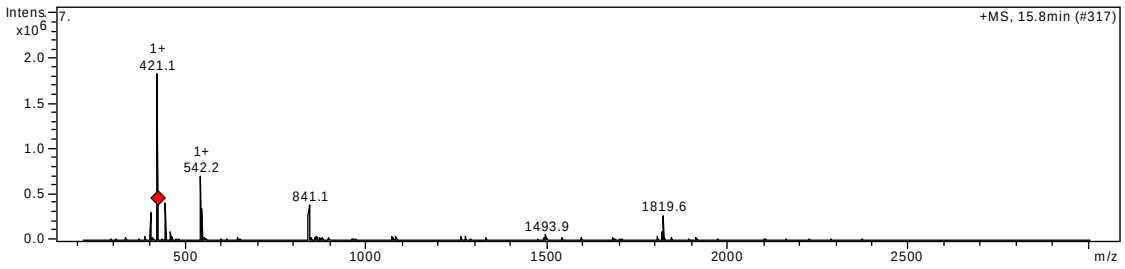


Figure 21: MS spectrum of compound no.5 found in the S-sample at 15.8min (t_r)

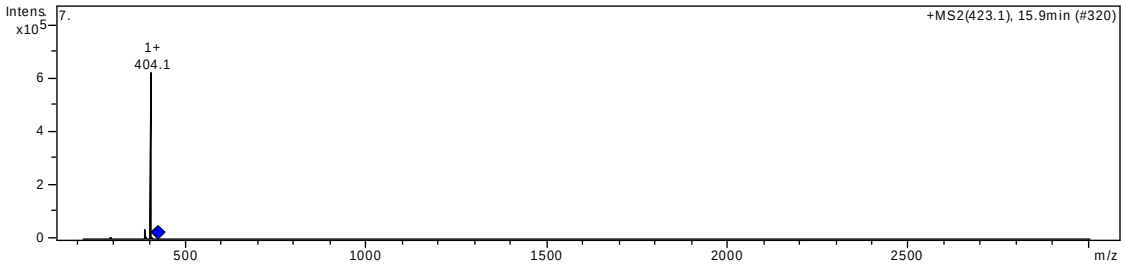


Figure 22: MS/MS spectrum of compound no.5 found in the S-sample at 15.8min (t_r)

With three precursor ions means that other ions will not be fragmented further even though these ions might be of importance for the identification. This is a disadvantage, since information might get lost when these peptide ions are in the same elution window as the chosen ions. MS/MS mode gives further fragmentation of a peptide detected and this can be used to identify the peptide and its amino acid sequence. With the fragmented mass spectrum,

peptide mass fingerprint searching engines can be used, and peptide identification can then be confirmed. The protein and peptide identifications of the samples will be discussed in 3.5.3.

The most important fact of a mass spectrum is that it can serve as a fingerprint of the molecule conveying information about its molecular mass, and when fragmentation occurs during ionization or by collision, fragmentation ions are useful structural information of the molecule. As mentioned earlier in 3.5.1, the possibility of obtaining information of separated compounds is present in LC-MS. The known mass of a peptide or a compound can be used to get an extracted ion chromatogram of the mass applied. In this way the peak with the mass of interest is extracted or isolated from the total ion chromatogram. Peptide peaks can be isolated and separated in the same way. However, when looking at the chromatogram for the reference glue and the S-sample, there are so many compounds. When a mass is applied to get extracted ion chromatogram, the extracted chromatogram obtained from this does not show only one peak but several of them. An extracted ion chromatogram (EIC) of the mass 553 is shown in Figure 23.

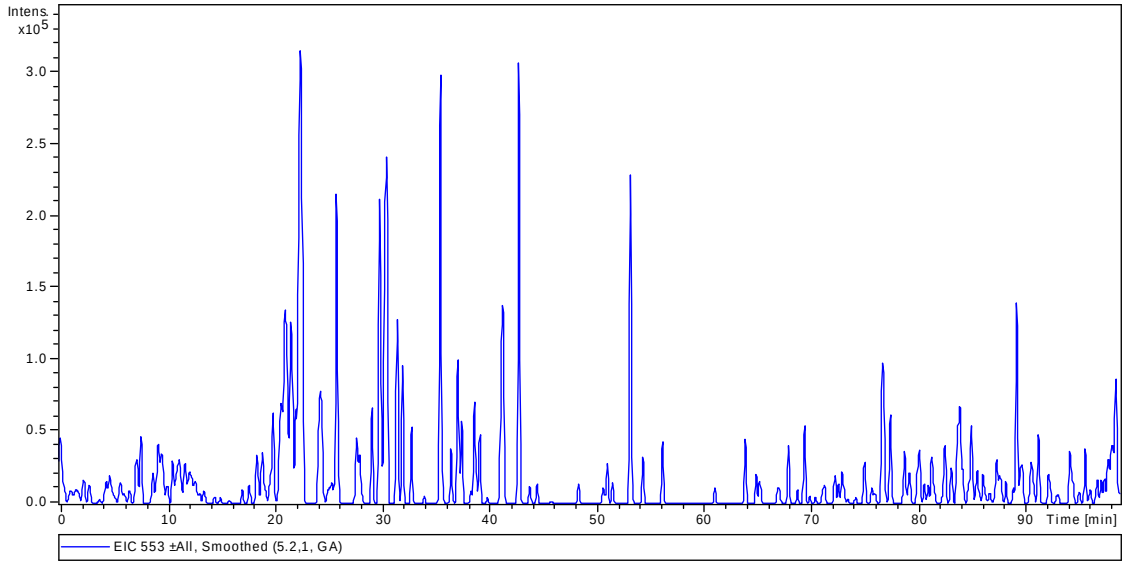


Figure 23: Extracted ion chromatogram of the mass 553 of the S-sample.

This makes it more complex, and due to the amount of the compounds in the sample and the masses they reveal, it is not easy to pick out which one to be further analyzed, now that most of the compounds are unknown. When doing mass scan over the whole range of the chromatogram to get masses and, a mass spectrum (to get an overview of the whole sample compounds), only a few masses were revealed, those with high intensity, but this does not

mean that they are masses of interested peptides. It is indeed necessary to do only mass scan over one peak at a time to get more correct mass information of the compound. However, within one peak in the chromatogram there might be more than one compound and this makes it a time consuming process to get all the peaks checked to find information about them. Additionally, information in the reference glue needs to be compared with that of the S-sample.

3.5.3 Data analysis and identification

Protein identification is mostly based on the electrospray MS analysis of peptides generated by proteolyic digestion. As mentioned before, the most widely used enzyme is trypsin. The advantage of using this enzyme is that every peptide other than the protein C terminus has at least two sites for efficient protonation, the N-terminal amino group and the C-terminal basic residue, so peptides are readily ionized and detected as positive ions by electrospray ionization.

Though trypsin cleaves exclusively C- terminal to arginine and lysine residues [14]; when the proteins in the reference glue sample and S-sample were of mixed origin and unknown protein compositions, peptides generated from these digestion are in large numbers, thus make the identification process more difficult, and time consuming data analysis work needed. Besides, the databases for protein are developing and still not complete deposited protein information bases .The match and probability scores system in e.g. Mascot has also been considered as too strict [14], though modification in the searching process might add false positives. Another fact is that when the detected compounds by MS/MS from the reference glue sample and the S-sample are 500 and more compounds, the maximum compounds that can be submitted to one Mascot search (free software version) is 300 compounds. Thus other options in the search have to be changed to meet the compound limitation in the search. One possibility is to change the intensity threshold in the data analysis before submitting to Mascot search or by submitting only a fraction of the data. However, when changing the intensity threshold, compounds that are excluded might be the ones that play a key part in the protein identification. This also is the case when only fraction of the chromatogram is submitted, leaving out peptides that might be an important part in matching the protein identification.

To limit the compounds and mass list in the chromatogram of the S-sample, the intensity threshold was set to 500000 and 287 compounds in the scan from 14-67 min were obtained. When the 287 compounds mass list was submitted to Mascot search engine for protein identification, the search results showed no significant protein Figure 24. The probability scores were low, the top score was 21 (for gi|119902094, predicted: similar to T cell receptor alpha chain (Bos Taurus), the score of 71 or higher would be significant for the match in this search.

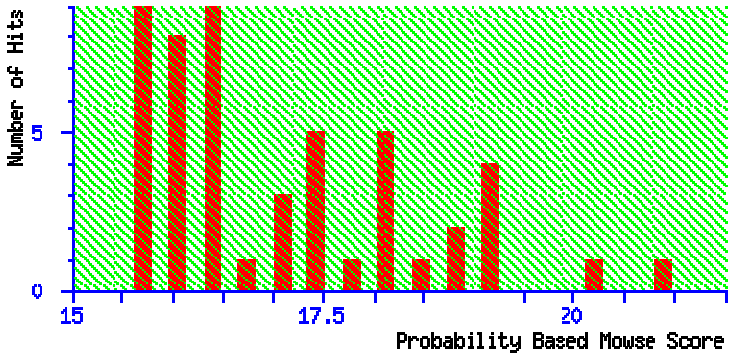


Figure 24: Mascot search results for the S-sample.

Another search for the reference glue showed better scores which were outside the green square (Figure 25). But the protein match were the Heat shock protein 70 (Fragment)-Trypanoplasma borreli, a bacteria protein and sequence coverage was only 8%. However, the peptide sequences SQVFSTYADNQPGVHIQVYEGER and MYQAGGGGGMPGGMPGGMPGGMPGGMPGGIPGG were found for the masses 875.75 and 961.30.

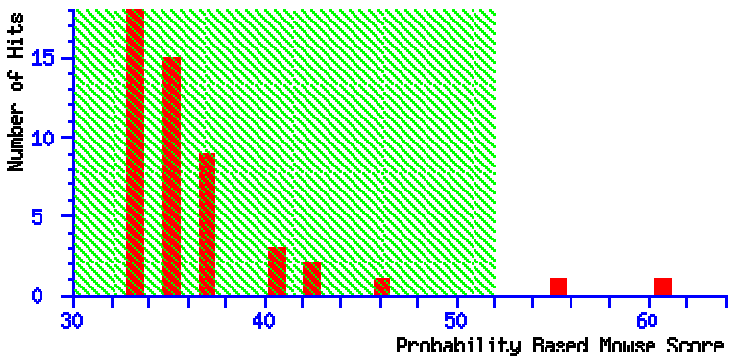


Figure 25: Mascot search for the reference glue.

Some of the suggested sequences from the data search engine may have very high scores, but still does not necessary give the true peptide sequence. One possible way to overcome this problem is to validate the results from one search engine by using suitable software and manually check the results. This software can verify the search engine suggestion by comparing the experimental MS/MS spectrum to the suggested sequence fragmentation. In this way the sequence will gain additional score and increase the confidence of the suggested sequence. In the present study, BioTool 2.2 software has been used for manual evaluation of some MS/MS spectra.

Peptide sequences that were found in the reference glue (listed in Table 8.) were not submitted to further identification at that time. However, when studying the S-sample, some peptides found were submitted to identification and confirmation. The compounds in the reference glue and the S-samples were not all peptides, the mass spectra as an example to this were shown in Figure 21 and Figure 22 in 3.5.2. MS and MS/MS spectra of compound no.25 (553.8) of the S-sample were submitted to identification since the spectra looked like a peptide, the MS/MS showed masses that are above its own mass marked with the blue dot.

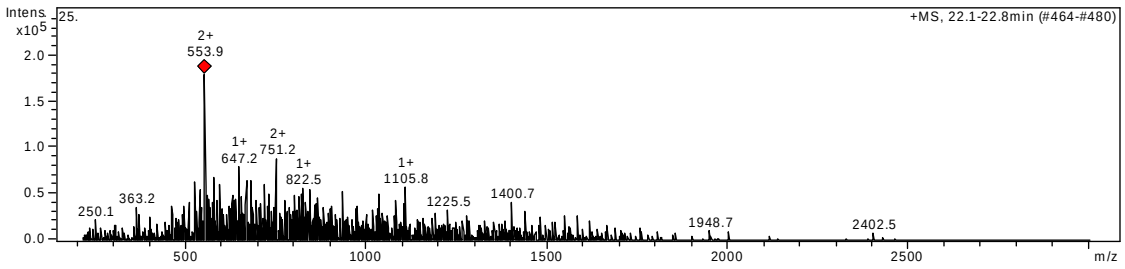


Figure 26: MS spectrum of compound no.25 found in the S-sample at 22.1-22.8 min (t_r)

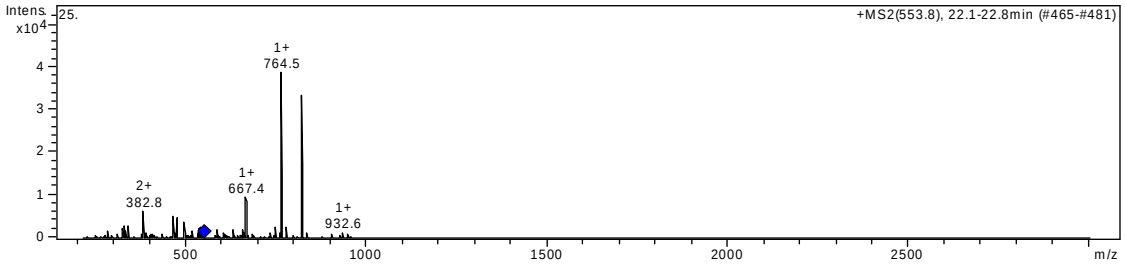


Figure 27: MS/MS spectrum of compound no.25 found in the S-sample at 22.1-22.8min (t_r)

The MS/MS spectra was exported to BIOtools, all the peaks from the spectrum has to be marked red manually before it can be submitted to the search (Figure 28)

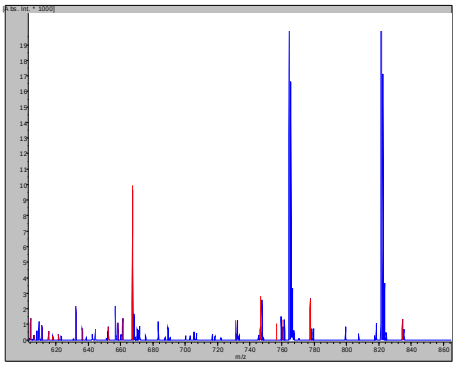


Figure 28: BIOtools window of the exported MS/MS spectrum.

The peptide were found to match the spectrum of the S-sample was chain A, the structure of collagen type I, single type I collagen molecule and the peptide sequence was GVEGPIGPAGPR. It was not identified in the search results the kind of animal (s) this collagen protein belonged. This sequence was then copied to the sequence editor in BIOtools and submitted for confirmation. More than 5 y and b were found in this match (Figure 29) and showed an example for the assigned peptide sequence GVEGPIGPAGPR, possibly originated from the protein suggested. And Figure 30 shows where the a, b and y are assigned in a peptide chain. In this way peptide identification can be confirmed and this could be applied to other peptide sequences that are found in the samples. However, hundreds of compounds were found in the S-sample and the reference glue sample. Due to the time limited in this thesis, this investigation could not be done.

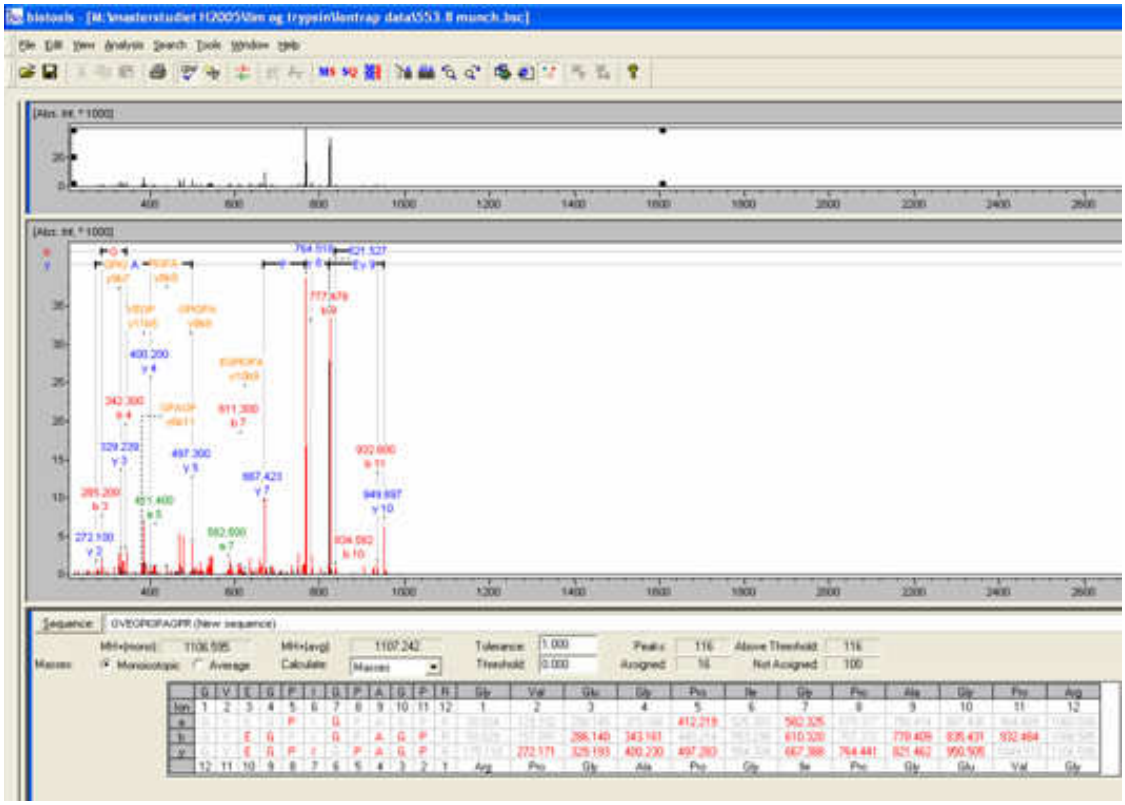


Figure 29: Biotool window, presents the raw MS/MS spectrum overlaid to the first two databased suggestion for peptide structures. Bottom table are the list of the corresponding a-, b- and y-ions.

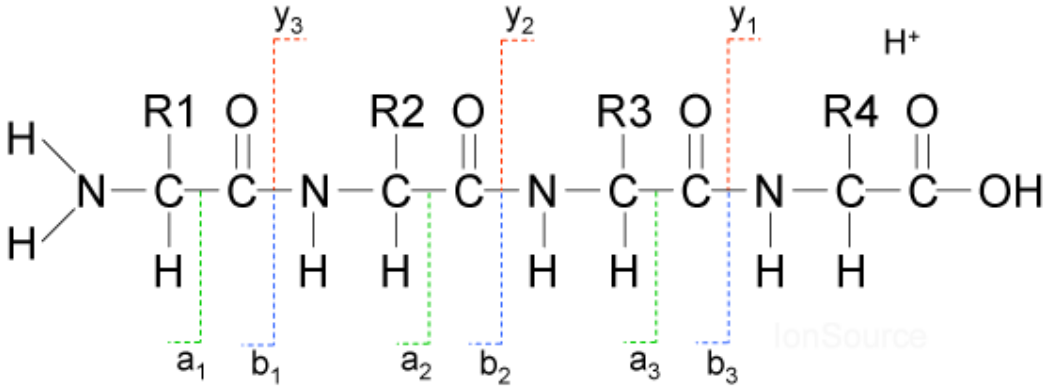


Figure 30: figure showing the a, b and y in the peptide chain(http://www.ionsource.com/tutorial/DeNovo/DeNovoTOC.htm)

In case of a single trypsinated protein, the results from the Mascot search engine for protein identification are more reliable as discussed in 3.4.1.

4. CONCLUDING REMARKS

The approach which is utilized in the present study shows that identification of peptides and peptide sequences from the target sample is possible by MS. However identified peptide can originate from quite many proteins which make the identification of one particular protein difficult. In this study protein identity confirmations were not possible, even though the tryptic digestion was proved to function well in the method.

For better identification of the proteins in the samples, it would be necessary to isolate the proteins one by one from the sample prior to trypsination and submission of peptide mixtures to LC-MS analysis. Such protein separation can be done by LC or 2D gel electrophoresis. The MS/MS spectra obtained can then be used in database searching for identification. In this way, the 300 compounds limitation in the Mascot search will not be a problem. Furthermore, the submitted peptides is generated from one protein which was isolated, thus the protein found in the database match is more certainly confirmed.

However, both the reference glue sample and the S-sample have unknown protein composition, and the amount of S-sample available was very limited and not sufficient for optimization of the separation parameters. Besides, the S-sample contained not only proteins, but also colour pigments and even cardboard materials, these have to be removed as well. This might be a more difficult, time and sample consuming process. Thus, the approach used in this study for characterizing the unknown samples was done in the possible way of available time and sample, and the information obtained here confirmed that the S-sample contain proteins similar to the reference glue, but not identical.

PART TWO

This second part of the thesis is not connected with the previous part of the present thesis. In this study, experiments were done to investigate some new aspects for Liquid Chromatography- Solid Phase Extraction- Nuclear Magnetic Resonance (LC-SPE-NMR) analysis method. The coupling of liquid chromatography and NMR, combined with the use of SPE, is gaining popularity due to technological improvements, but the coupling still has many shortcomings to be overcome. The following describes both basics in the LC-SPE-NMR method, some performed experiments, as well as results and discussions concerning the subject.

5. INTRODUCTION

The coupling of Liquid chromatography with Nuclear Magnetic Resonance spectroscopy (LC-NMR) and mass spectrometry (LC-MS) have been used in the last two decades to solve many complex analytical problems [41-43]. Analytes are separated in the liquid chromatography system and then detected by NMR spectrometry or MS. NMR analysis of a compound often requires sample amounts in the microgram and milligram. Therefore, analytes at low concentration need to be concentrated in order to be analysed by NMR. The commercial LC-NMR systems do include a solid phase extraction (SPE) unit that is used to trap and concentrate the analyte from the LC system, however, the commercial SPE system is not well fitted for combination with the LC system and it has also been observed that the materials in the commercial SPE columns have limited trapping capacity [44]. Therefore, an improved on-line method for concentrating analytes from the LC column before NMR analysis is necessary and attempts in this direction were tested.

Liquid chromatography is a widely used and robust separation technique, and NMR is probably the most important tool for structure determination. Thus, the coupling of these two techniques together makes a powerful analytical tool for determining the structures of unknown organic compounds in complex samples. NMR analysis is relative insensitive, but the combination of LC-NMR with solid phase extraction (SPE) can enable sample enrichment, thus analytes at low concentration can be detected and structure elucidated.

5.1 NMR spectroscopy

Over fifty years since the first observations of Nuclear Magnetic Resonance (NMR) were made in solids and liquid samples, NMR spectroscopy today represents the most versatile and informative spectroscopy technique applied for structural and conformation analysis. Samples can be examined with one-dimensional (1D) and two-dimensional (2D) NMR experiments. Three or four-dimensional NMR (3D and 4D) spectra can in fact also be obtained, although this rather specialized subject will not be discussed here.

Chemical environment influences the resonance frequency of a nucleus, and one nucleus can influence the resonance of another through intervening chemical bonds. NMR spectra and chemical shift, spin-spin coupling to ^{13}C or ^{15}N, ^{1}H-^{1}H coupling, ^{1}H-^{1}H splitting patterns, ^{13}C -^{13}C coupling etc. can provide important information to obtain partial or complete structure determination of unknown organic compounds. But NMR is very insensitive relative to other techniques such as Infrared and Ultra Violet spectroscopy [45]. In UV similar chromophores add up one signal intensity. In NMR each atomic position is giving a signal so increasing the molecular weight does not improve S/N as in UV. The molarity of the sample is important. Different NMR experiments require different amounts of sample. A two-dimensional long-range proton-carbon correlation experiment obviously requires more sample than a straightforward proton experiment. NMR spectroscopy is the only method, among various spectroscopy method, that is capable of providing the full information needed for rigorous structure determination of a compound e.g. natural product, including determination of its relative stereochemistry and discrimination between positional isomers [46].

5.2 LC-NMR

Watanabe and Niki performed the first on-line LC-NMR experiments in the late 1970s [47]. Their work demonstrated stopped-flow measurements of a mixture of known compounds. LC is easily coupled with UV detectors and mass spectrometry (MS). But coupling with NMR has proven to be more difficult [41]. One of the problems is the difference between the amount of sample required to obtain NMR spectra and the capacity of the LC system [41]. Further more, the LC peaks are often of larger volume than an LC-NMR cell, so not the entire available sample is used [41]. Broadening of the peaks in the flow system can also be a problem so that not the entire peak is used [41]. However, several technological advances that have been made in recent years, such as solvent suppression methods [48] and improvements on probe design [42] have increased the potential and stimulated the interest in LC-NMR hyphenated technique. LC-NMR has been used as an analytical technique for the study of e.g. drug impurities [41], drug metabolites [49], sugar nucleotides [50], and natural products [43].

5.2.1 Modes of operation for LC-NMR

The connection between the LC system and the NMR flow cell can be done on-line with polyether ether ketone (PEEK) tubing or fused silica capillaries [42]. The LC system can be as close as 30-50 cm to the NMR magnet when the magnet is shielded [42]. The distance between the LC and NMR is increased to 1.5-2 m when the magnet is unshielded [42]. To monitor the chromatography and trigger the peak of interest, a UV detector is usually used in the LC system [42]. There are different modes of operation for LC-NMR, on-flow (continuous flow), stop- flow, loop collection and solid phase extraction (SPE) collection [42].

On –flow LC-NMR

In this mode, the NMR spectrometer acts similar to a UV and MS detector in a chromatographic system; the analytes are measured without stopping the flow. The outlet of the chromatography system is connected to the NMR detection cell. The NMR spectra are acquired continuously while the sample is flowing through the detection cell. Figure 31 shows an example setup of this mode.

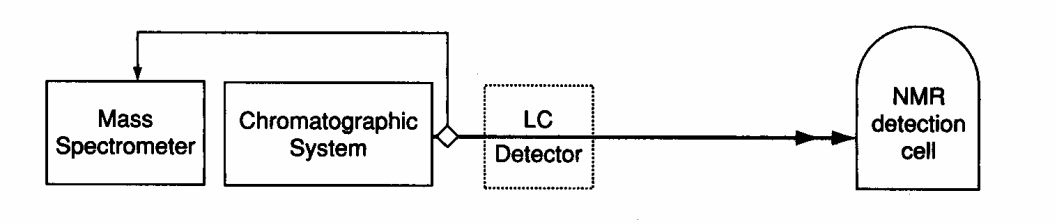


Figure 31: Schematic set-up of the on-flow mode in LC-NMR and LC-NMR/MS

On- flow experiments require more samples for analysis because the residence time in the NMR flow cell is very short during the chromatographic run [42]. Short residence time within the radio frequency (RF) coil often results in a poor signal to noise (S/N) ratio for the NMR spectra [43]. Signal to noise ratio measurement in which the peak high of the analyte is compared to the noise level in the spectrum is a sensitivity matter of the NMR instrument. Decreasing the flow rate with a factor of 3-10 increases the residence time and the S/N ratio for each compound [43]. On-flow mode allows rapid screening of 1D proton spectra, however, only for the major peaks from the chromatographic separation [43].

Stop-flow LC-NMR

In this mode, the eluent is directly flowing from the chromatographic system into the NMR probe. NMR measurement is carried out under no flow or static condition by the use of a valve to stop the elution when the analyte reaches the flow cell volume within the RF coil. Only selected peaks are measured by the NMR spectrometer. The separation is monitored in parallel with a LC detector e.g. a UV detector. The separation is interrupted after a certain time delay that is necessary to allow the peak to move from the LC detector to the NMR detection flow cell. Figure 32 shows an example of the coupling.

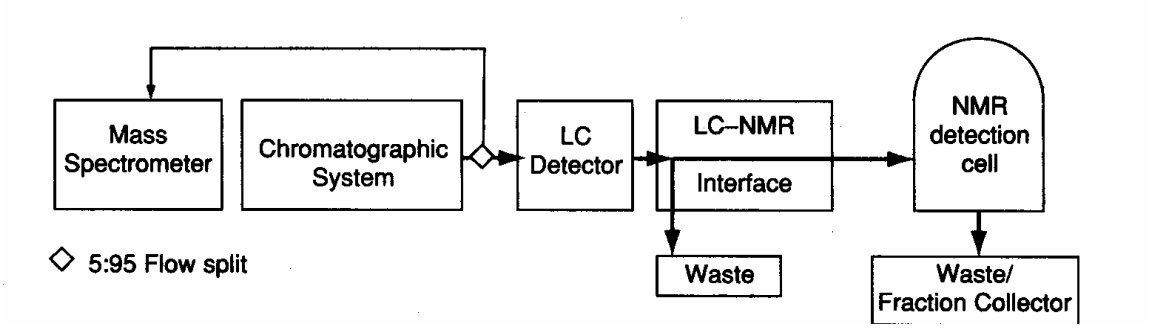


Figure 32: Schematic set-up of the stop-flow mode in LC-NMR and LC-NMR/MS

It is necessary to calibrate this delay time which is dependent on the flow rate and the length of the tubing connecting the LC with the NMR [42]. Separation is continued until the next peak is positioned in the NMR detection cell when the chromatographic run is stopped when the analyte peak is in the flow cell [42] resulting in a set of NMR spectra for the selected compounds.

A stop- flow experiment requires that the retention time of the analyte is known [51]. The method is difficult to apply when the eluting peaks are close such as in a complex crude plant extracts when compounds are present in small amounts (g); hours of acquisition time are required [51]. The frequent stops may also disturb the quality of the separation, and highly concentrated compounds may pollute the NMR detection cell causing memory effect [43]. However, the stop- flow mode is much more sensitive than the on- flow mode [51] and all kinds of 1D and 2D NMR measurements can in principle be carried out [51, 52].

Loop collection LC-NMR

A storage loop is used to collect the eluent directly from the chromatographic system without interrupting the separation [42]. Analyte peaks can be trapped in the subsequent storage loops. Loop collection can be used to collect more than one peak of interest in the same chromatographic separation. After the separation is completed, the loop contents are transferred sequentially into the NMR spectrometer for further analysis as shown in a setup of this mode (Figure 33).

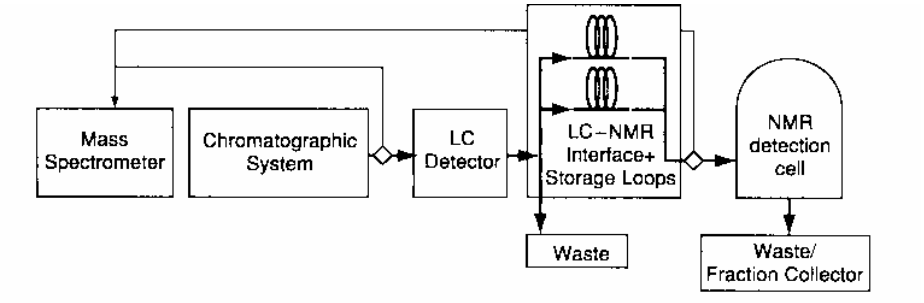


Figure 33: Schematic set-up of the loop collection mode in LC-NMR and LC-NMR/MS

All kind of 1D and 2D NMR measurements can be carried out [52]. However, the delay time between the LC detector and the loop storage device and the delay time for transport from these loops to the NMR flow cell must be calibrated [43]. The analytes must also be stable inside the loops during the extended period of analysis.

SPE and LC-SPE-NMR

The traditional use of solid phase extraction (SPE) in analytical chemistry is a preparative step for sample clean up and/or enrichment before chromatographic analysis. In LC-SPE-NMR, SPE is used to trap and concentrate analytes after liquid chromatography separation (Figure 34).

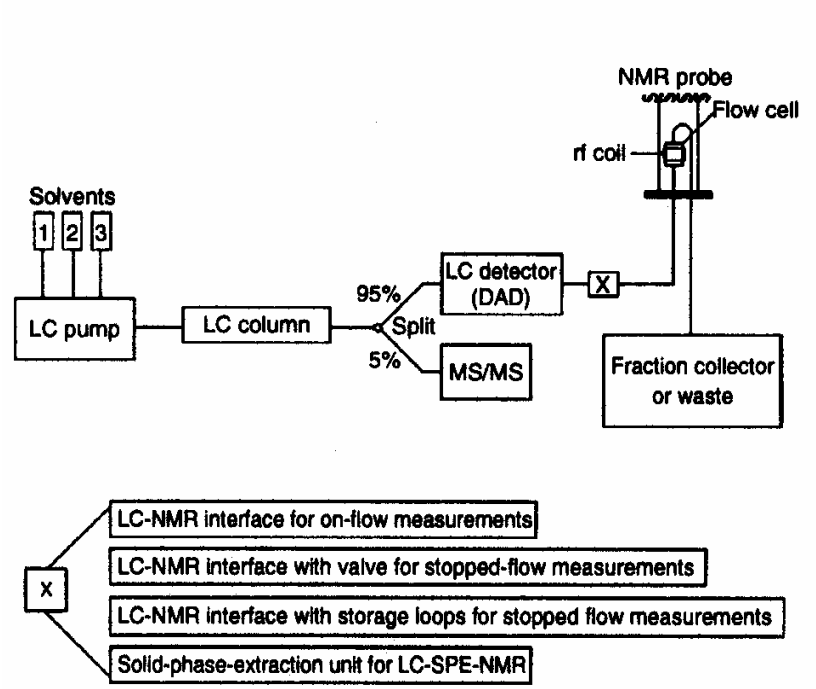


Figure 34: Schematic set-ups of different LC-NMR modes including Solid-phase-extraction unit for LC-SPE-NMR system [43].

Solid phase extraction of analytes from the LC separation with sequent NMR analysis has been carried [53-57]. The LC-SPE-NMR coupling allows increase of the sensitivity compared to a conventional LC-NMR by a factor of at least 2 [53]. The peaks of interest from the analytical LC-column are trapped in the SPE cartridges without any elution from the cartridges while in contact with the mobile phase [56]. The compounds are extracted to the chosen solid phase material that acts as a sorbent [54]. The trapping procedure can be a single or multiple trapping of the compounds on the cartridges to achieve the wanted sample amount [56]. The solvents and buffers that are in used for separation can be removed and the concentrated compounds then dissolved in a suitable solvent prior to NMR analysis [54]. In this way nondeuterated solvents can be used in the LC-SPE step, saving cost and also prevent the exchange of acidic protons in like –OH, -NH, $-NH_2$ with D_2O.

Transfer of water in the mobile phase from LC system into the NMR is not wanted. This water usually gives solvent suppression in NMR, in such case, analyte signals of interest will not be observed [42, 48]. An approach to solve this problem is to have a column to absorb the unwanted water. The column can be used in the connection between the LC and the NMR, either in off- line or on-line LC- NMR. Traditionally $MgSO_4$ is used in organic

chemistry to get rid of water in many chemical experiments. A column packed with $MgSO_4$ might solve the water problem in NMR, however, such column is not commercial available, and has not been investigated for removal of water in an LC-NMR system.

Solvents in LC-NMR system

In LC-NMR systems both nondeuterated and deuterated solvents are used to carry out analysis. NMR spectra are preferably acquired in deuterated solvents, but use of deuterated solvents for HPLC is expensive and of limited availability. Choice of solvent and its purity is very important in NMR to obtain good NMR spectra and to avoid solvent suppression. $DMSOd_6$ is often used in NMR. The possibility of using DMSO in the LC system is therefore of interest. In the LC system a UV detector is often used, and this raised the interest of knowing the DMSO UV absorbance. DMSO is also more viscous than regular HPLC solvents such as MeOH and ACN. The DMSO viscosity is 2.24 cP while the viscosity of ACN is 0.34 cP. Hence, there may be pressure problems and pump instability using DMSO as mobile phase solvent. A HPLC pump usually has a pressure limit of about 400 bar. Furthermore, in a common LC system, mobile phase solvents are often degassed with helium or by ultra sonic treatment. Such treatments of solvents in the LC part might give rise to impurity signal(s) in NMR.

5.3 Aim of the study

The new aspect, which was aimed to investigate in this part, is how the solvent dimethyl sulfoxide works as a LC mobile phase in a LC-NMR system. Additionally it was of interest to see if a $MgSO_4$ column could be used to remove water from the solvents used on a SPE column in a LC-SPE-NMR system.

6. EXPERIMENTAL

6.1 DMSO UV absorbance measurement

A UV-Visible Spectrometer from Varian, Cary 100 Bio was used with quarts cuvettes to measure the UV absorbance. Solutions of 1 % DMSO in ACN was prepared. DMSO was bought from Merck (Germany) and HPLC grade ACN from Rathburn (Walkerburn, UK). ACN was used in the reference cell, and the 1 % DMSO solution was scanned with baseline correction.

6.2 Pressure measurement with DMSO as mobile phase

A Hitachi L-7110 isocratic LC pump was used, and a Hotsep Tracy, 1.0 mm× 5 mm, Hypercarb column (5 µm particle size, 100 Å pore size) from G&T Septech (Kolbotn, Norway). Nondeuterated DMSO from Merck and HPLC grade ACN from Rathburn were used, and the column pressures using DMSO and ACN were measured at different flow rates.

6.3 He degassing of deuterated solvents and ultrasonic treatment

Deuterated water (D_2O) (D: 99.96%) and deuterated acetonitrile (CD_3CN) (D: 99.8%) were obtained from Cambridge Isotope Laboratories, Inc (Andover, MA, USA). Deuterated water and acetonitrile were degassed with helium and ultrasonic treatment in for 10 minutes in both cases, before proton spectra of the solutions were acquired. Solutions of the same D_2O and CD_3CN without treatment were included in the NMR acquisitions as references spectra. NMR spectra were acquired on a 300 MHz NMR instrument (Bruker DPX 300), solvent locked on D_2O, and CD_3CN, with 128 scans.

6.4 $MgSO_4$ as SPE column

6.4.1 Column packing process

$MgSO_4$ solid particles (Nowegian MedicalDepot AS (NMD)) was first sieved through a 36 µm and a 25 µm pore size sieve (Retsch test sieve, Retsch GmbH, Germany), then dried at 150º C for at least 12 hours to get rid of water in the material, cooled and kept in an exiccator with drying agent Orange gel from Merck (Darmstadt, Germany). The $MgSO_4$ water content (18 %) was obtained by drying 2.00 g $MgSO_4$ at 150º C overnight and by calculating the weight loss (0.36 g) due to water evaporation. A steel column 0.3 mm I.D.x.30 mm was slurry packed, the $MgSO_4$ which was sieved through the 25 µm was used, and CCl_4 as slurry solvent. A Hitachi L-7110 isocratic LC pump from Merck (Darmstadt, Germany) was used to pack the column using the downward pressure liquid slurry method which is developed in house. The packed column was then dried at 150° C for at least 12 hours and kept in the exiccator until further use.

6.4.2 Testing the MgSO4 column

The $MgSO_4$ column was tested in a LC-UV system. The system consisted of a Hitachi L-7110 isocratic LC pump, a 6- port Valco valve and a 50 µL loop, a Tracy Hypercarb column, 1.0mm I.D. x 5mm, and Spectra 100 UV detector equipped with a 100 µm I.D. on-capillary flow cell. The mobile phase consisted of ACN / water (50/50, v/v), and the flow rate was 5 µL/min. A solution (0.5 µL) of ACN and water (50:50 v/v) was injected in the LC system without $MgSO_4$ column and this was repeated with the $MgSO_4$ column coupled ahead of the Tracy column.

7. RESULTS AND DISCUSSION

7.1 DMSO UV absorbance

DMSO has UV absorbance that is suitable in a LC-UV system. The UV scans of both nondeuterated DMSO and deuterated $DMSOd_6$ examined Figure 35 and Figure 36, were within the wavelength of the UV detector which is between 200 – 400 nm. Due to high UV absorbance of DMSO (Figure 35) and $DMSOd_6$ (Figure 36), at low wavelengths UV detection can only be used above approximately 245 nm.

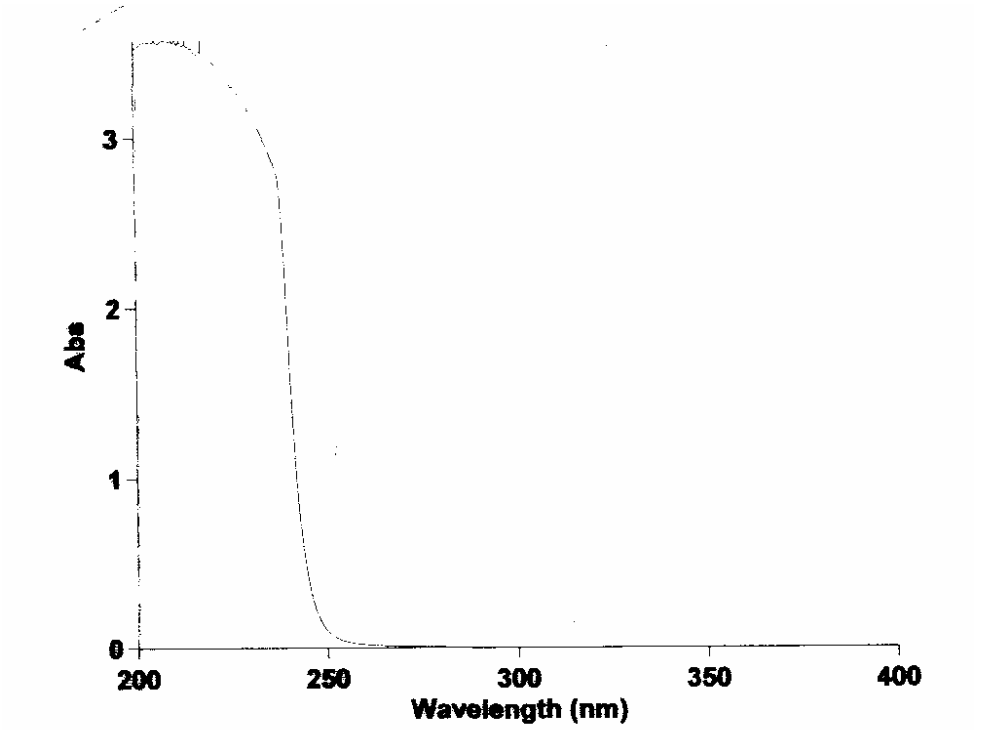


Figure 35: UV scan of DMSO

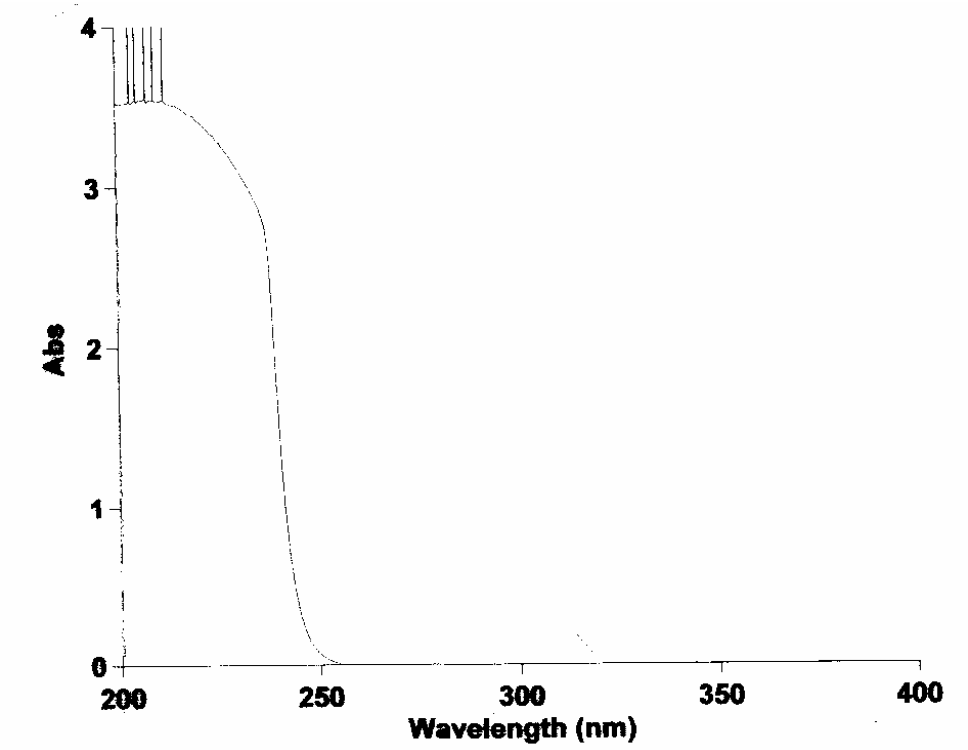


Figure 36: UV scan of $DMSOd_6$

7.2 DMSO as mobile phase

The pressure of DMSO was measured as described in 6.2 and the pressure obtained is presented in Table 9 and a plot of the pressure as function of the flow rate is shown in Figure 37. DMSO can be used as LC mobile phase.

Table 9: Column pressure using DMSO and ACN and Hypercarb column.

Flow rate (µL/min)	**Pressure (bar) DMSO**	**Pressure (bar) ACN**
30	14	0
60	30	1
100	57	5
150	94	9

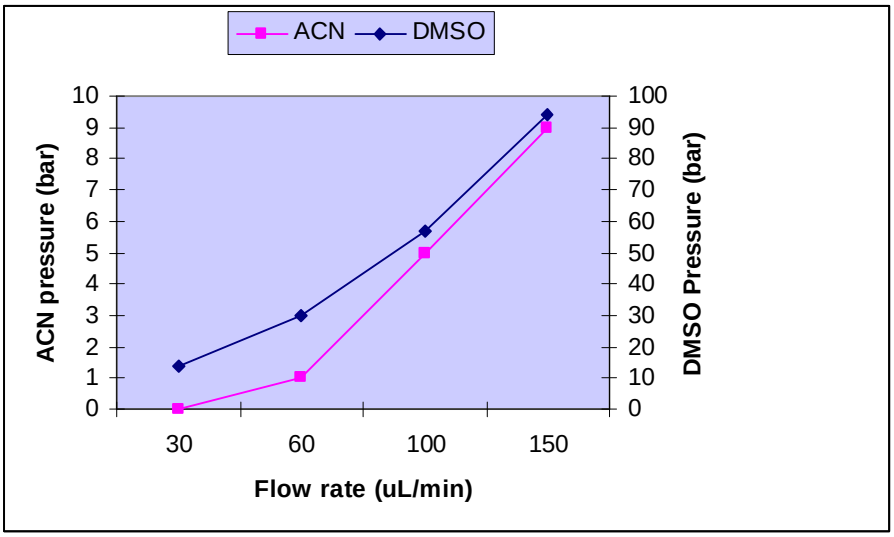


Figure 37: Plot of DMSO and ACN pressure as a function of flow rate

7.3 Deuterated solvents and treatments

When deuterated solvents D_2O and CD_3CN were He degassed and ultrasonically treated, NMR spectra (Figure 38, Figure 39) showed no impurity signals in the treated solutions and thus it is concluded, that deuterated solvents can be degassed either by He or by ultrasonic treatment.

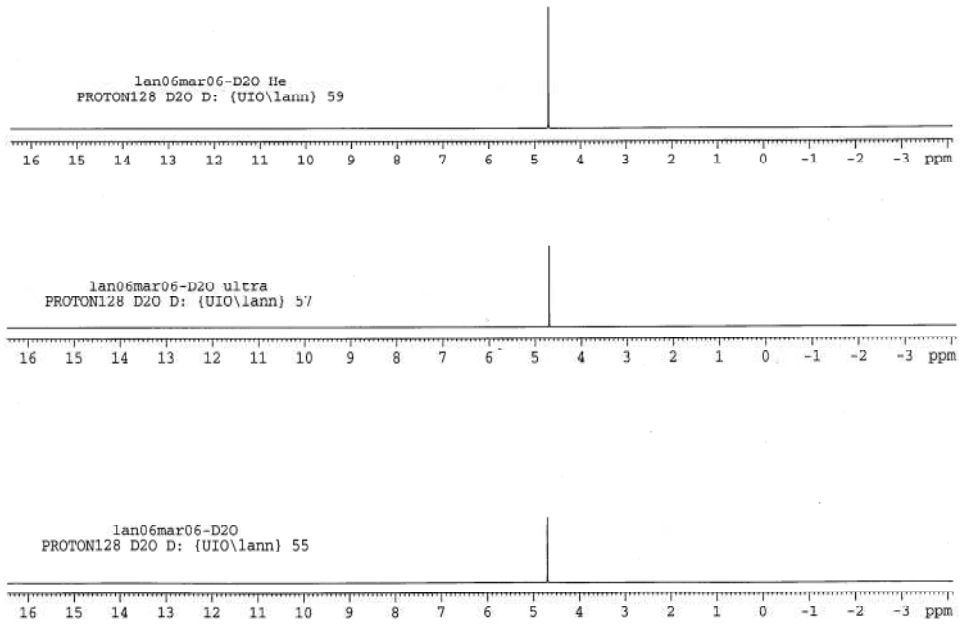


Figure 38: NMR spectra of D_2O without treatment at the bottom, with ultrasonic treatment in the middle and with helium degassing on the top. solvent peak, a singlet at 4.70 ppm.

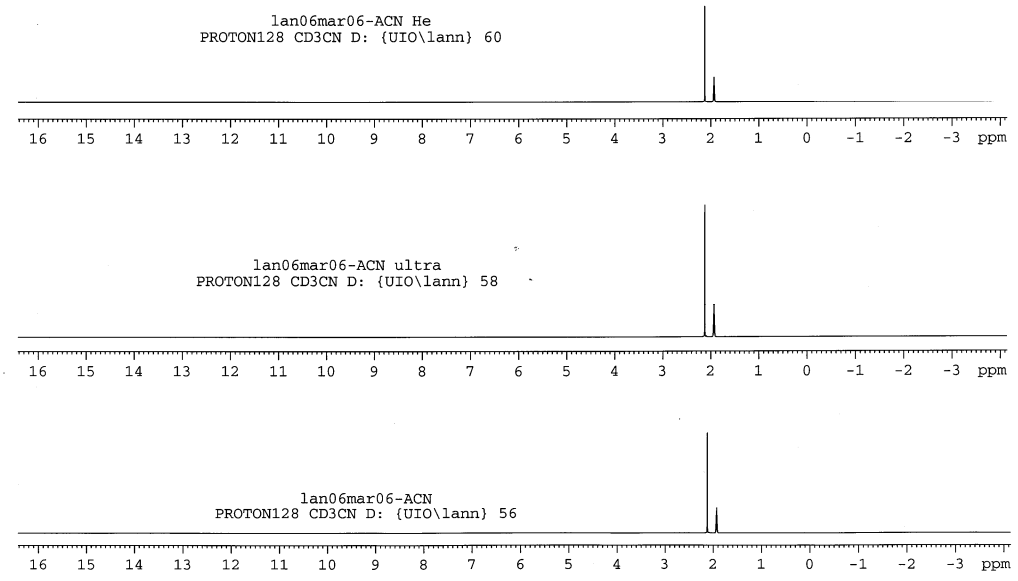


Figure 39: NMR spectra of CD_3CN without treatment at the bottom, with ultrasonic treatment in the middle and with helium degassing on the top. Solvent peaks a quintet at 1.96 ppm and water peak of the solvent a singlet at 2.15 ppm.

7.4 $MgSO_4$ column

Different procedures for getting rid of water contamination in the slurry were tested e.g. filtration, and drying with molseive. Proton NMR acquisitions of these solutions showed water signal and also other contaminations. ACN as slurry solvent with $MgSO_4$ particles was also tested, but CCl_4 give better homogeneity slurry with $MgSO_4$ (Figure 40). The water from the CCl_4 solvent would vapour when the column was dried after packing. The column was tested in the LC system as described in 6.4.2. The chromatograms of the testing show in Figure 41 that the water peak is smaller and moved when the $MgSO_4$ column were used in the system.

Figure 40: Slurry of $MgSO_4$ with ACN (on the left) and CCl_4 (on the right)

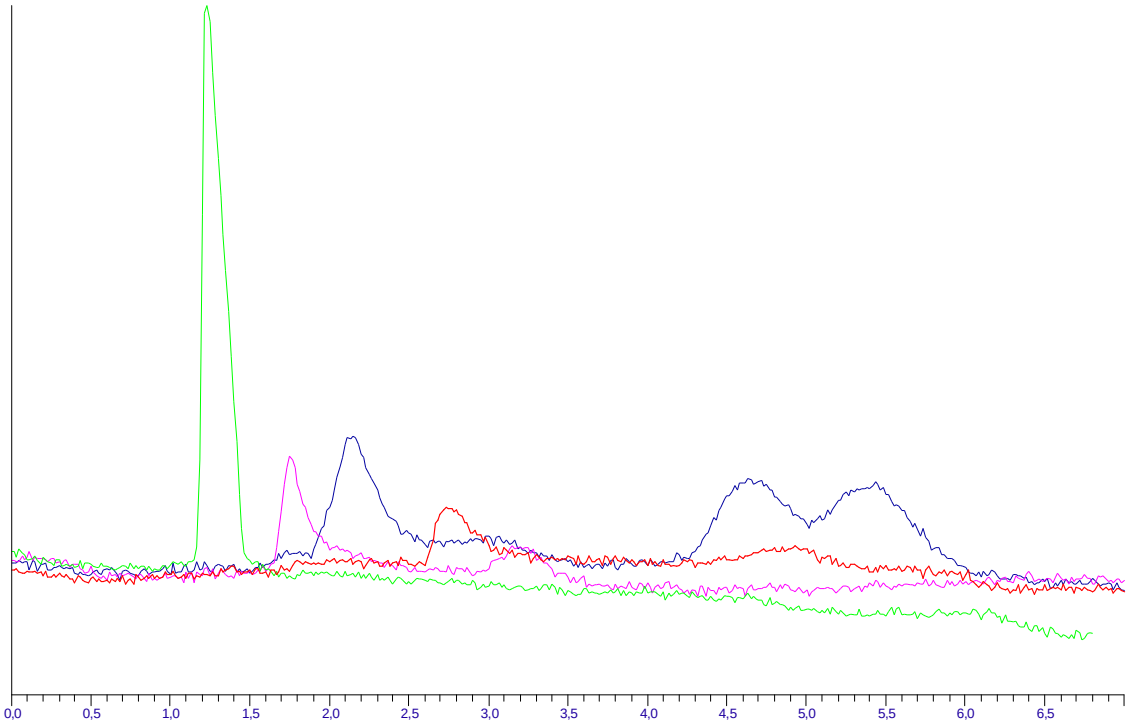


Figure 41: The green is the water peak without $MgSO_4$ in the system. The red, blue and pink peaks are those with the $MgSO_4$ column in the system.

8. APPENDIX

8.1 Mascot seaching procedure ion trap MS

Data analysis

1. Choose “Open” in the Data analysis window.
2. Open folder and choose the desired analysis file.
3. Check and mark “TIC ± All.”
4. Choose Find in the Toolbar → parameters:

a) AutoMS (n) parameters
- Intensity threshold: variable (50000-1000000)
- Retention time window (min): 0.75
- Spectrum type (Line/Profile): Profile spectra only
- Background subtraction (MS only): None
- Uncheck “Create individual AutoMS (n) chromatogram traces.”

b) Mass List parameters →Choose Apex page
- Peak width (FWHM) (m/z): 0.1
- S/N threshold: 0.1
- Relative intensity threshold (base peak): 0
- Absolute intensity threshold: 0.1
- Choose “Use peak finder to calculate peak position.”

c) Charge Deconvolute parameters
- Peptide / small molecules
- Adduct ions: +H (first window) and –H (second window)
- Deconvolute: Full scan, MS MaxRes Scan, MS (n)

Low mass:	215	100	215
High mass:	3000	3000	3000
Abundance cut-off (%):	5		1
Resolved-isotope deconvolution			
Maximum charge:	3	3	2

- Uncheck “Related-ion deconvolution (MS full scan only).”

d) Mascot export options parameters:
- “Set global charge limitation” (2+ and 3+)
- “Mixed list (non-deconvoluted and deconvoluted)
- Unchecked “Prefer full scan spectrum deconvolution results to MaxRes results”

Press OK!

5) Choose Find in the toolbar → Compounds- AutoMS (n)
6) Select and mark “Compounds mass spectra” so it is highlighted
7) Masslist in the toolbar → Find
8) Deconvolute in the toolbar → Mass Spectra
9) File in the toolbar → Export → Compounds and save as mgf file (MS search) and as bsc file (MS/MS search).

Bio Tools

Open Bio Tools
File → Open the target file
Choose the MS or MS/MS icon at the toolbar
Taxonomy: All entries chosen (or the right family if known)
Database: MSDB (or NCBI)
Enzyme: Trypsin
Fixed modification:
Variable modification:
Peptide tolerance: ± 2 Da
MS/MS: ± 1.5 Da
Protein mass: leave open
Charge state: variable (+1, +2, +3)
Instrument: ESI-TRAP
Report top: variable (20-100 hits)
Press START
Data will be transferred to Mascot to obtain
Mascot searching results

Biotools sequence editor

-Open Sequence Editor in the upper Toolbar (Window Start Sequence editior).
-Click on the icon at the left in the upper toolbar and the sequence will automatically be copied in the sequence editor page. If the sequence is not copied in the Sequence Editor automatically, copy the sequence, but not the flang amino acids, and paste it in the page.
-To get information about b and y-ions, press Bio at the toolbar (both a, b and y ions will appear.

8.2 Mascot search results

Mascot search results standard protein beta-A

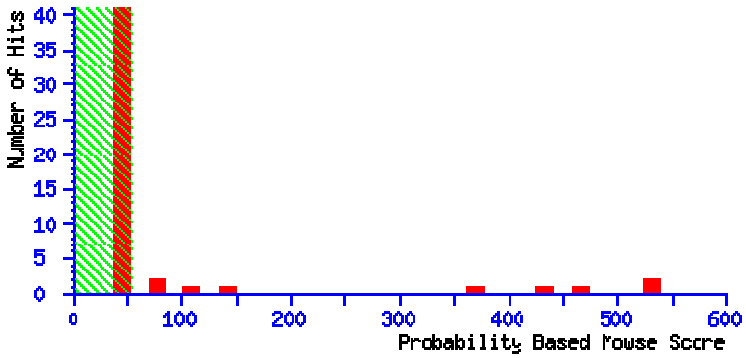
```
User            : Lan
Email           : lan.nguyen@kjemi.uio.no
Search title    :
MS data file    : DATA.TXT
Database        : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Timestamp       : 21 Oct 2007 at 15:08:51 GMT
```

Protein hits :	**1BSQA**	beta-lactoglobulin mutant YES - bovine
	1BSOA	bovine beta-lactoglobulin a - bovine

	LGBUI	beta-lactoglobulin - water buffalo

Probability Based Mowse Score

Ions score is -10*Log (P), where P is the probability that the observed match is a random event.
Individual ions scores > 52 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Mascot Search Results

Protein View

Match to: **1BSOA** Score: **518**
bovine beta-lactoglobulin a - bovine
Found in search of DATA.TXT

Nominal mass (M_r): **18641**; Calculated pI value: **4.76**
NCBI BLAST search of 1BSOA against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Bos taurus
Links to retrieve other entries containing this sequence from NCBI Entrez:
1BSY from Bos taurus
2BLG from Bos taurus
3BLG from Bos taurus

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **77%**

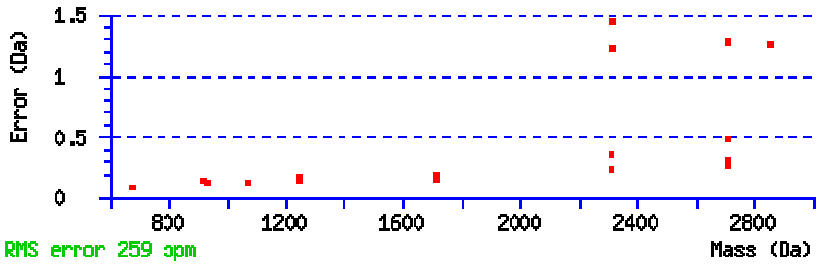
Matched peptides shown in **Bold Red**

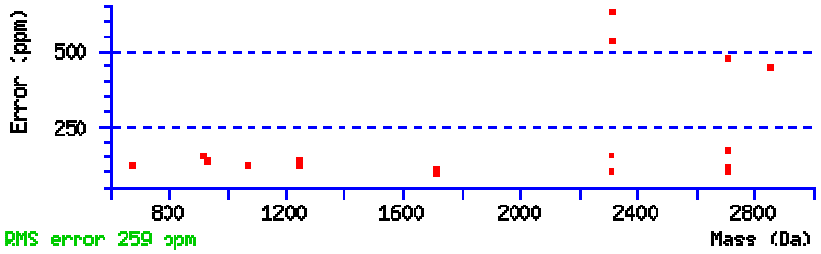
```
    1 LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP
   51 EGDLEILLQK WENDECAQKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK
  101 KYLLFCMENS AEPEQSLVCQ CLVRTPEVDD EALEKFDKAL KALPMHIRLS
  151 FNPTQLEEQC HI
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
1 - 8	933.6700	932.6627	932.5365	0.1262	0	- .LIVTQTMK.G (Ions score 37)
1 - 8	467.3400	932.6654	932.5365	0.1290	0	- .LIVTQTMK.G (Ions score 52)
9 - 14	673.4700	672.4627	672.3806	0.0821	0	K.GLDIQK.V (Ions score 28)
15 - 40	1354.3300	2706.6454	2706.3687	0.2768	0	K.VAGTWYSLAMAASDISLLDAQSAPLR.V (Ions score 108)
15 - 40	1354.3300	2706.6454	2706.3687	0.2768	0	K.VAGTWYSLAMAASDISLLDAQSAPLR.V (Ions score 70)
15 - 40	1354.3500	2706.6854	2706.3687	0.3168	0	K.VAGTWYSLAMAASDISLLDAQSAPLR.V (Ions score 107)
15 - 40	903.2900	2706.8482	2706.3687	0.4795	0	K.VAGTWYSLAMAASDISLLDAQSAPLR.V (Ions score 38)
15 - 40	1354.8300	2707.6454	2706.3687	1.2768	0	K.VAGTWYSLAMAASDISLLDAQSAPLR.V (Ions score 51)
15 - 40	1354.8400	2707.6654	2706.3687	1.2968	0	K.VAGTWYSLAMAASDISLLDAQSAPLR.V (Ions score 52)
41 - 60	1157.2500	2312.4854	2312.2515	0.2340	0	R.VYVEELKPTPEGDLEILLQK.W (Ions score 34)
41 - 60	771.8800	2312.6182	2312.2515	0.3667	0	R.VYVEELKPTPEGDLEILLQK.W (Ions score 36)
41 - 60	1157.7500	2313.4854	2312.2515	1.2340	0	R.VYVEELKPTPEGDLEILLQK.W (Ions score 38)
41 - 60	772.2400	2313.6982	2312.2515	1.4467	0	R.VYVEELKPTPEGDLEILLQK.W (Ions score 45)
84 - 91	458.8100	915.6054	915.4661	0.1393	0	K.IDALNENK.V (Ions score 59)
92 - 100	1065.7100	1064.7027	1064.5754	0.1273	0	K.VLVLDTDYK.K (Ions score 27)
92 - 100	533.3600	1064.7054	1064.5754	0.1301	0	K.VLVLDTDYK.K (Ions score 71)
102 - 124	1424.2800	2846.5454	2845.2907	1.2547	0	K.YLLFCMENSAEPEQSLVCQCLVR.T (Ions score 48)
125 - 135	623.3700	1244.7254	1244.5772	0.1482	0	R.TPEVDDEALEK.F (Ions score 61)
125 - 135	623.3700	1244.7254	1244.5772	0.1482	0	R.TPEVDDEALEK.F (Ions score 45)
125 - 135	623.3700	1244.7254	1244.5772	0.1482	0	R.TPEVDDEALEK.F (Ions score 60)
125 - 135	1245.7600	1244.7527	1244.5772	0.1755	0	R.TPEVDDEALEK.F (Ions score 31)
149 - 162	858.4900	1714.9654	1714.7985	0.1670	0	R.LSFNPTQLEEQCHI.- (Ions score 50)
149 - 162	858.4900	1714.9654	1714.7985	0.1670	0	R.LSFNPTQLEEQCHI.- (Ions score 34)
149 - 162	858.5000	1714.9854	1714.7985	0.1870	0	R.LSFNPTQLEEQCHI.- (Ions score 27)



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>P1;1BSOA
bovine beta-lactoglobulin a - bovine
N;PDB title: 12-bromododecanoic acid binds inside the calyx of bovine
beta-lactoglobulin
C;Species 1BSOA: Bos primigenius taurus (cattle)
C;Species 1BSY: Bos primigenius taurus (cattle)
C;Species 2BLG: Bos primigenius taurus (cattle)
C;Species 3BLG: Bos primigenius taurus (cattle)
A;Note: var. variant a, breast, mammary gland
R;Qin, B.Y.; Creamer, L.K.; Baker, E.N.; Jameson, G.B.
submitted to the Protein Data Bank, August 1998
A;Reference number: A77091; PDB:1BSO
R;Qin, B.Y.; Creamer, L.K.; Baker, E.N.; Jameson, G.B.
Febs Lett. 438, 272, 1998
A;Title: 12-bromododecanoic acid binds inside the calyx of bovine beta-
lactoglobulin.
A;Reference number: TN079969
C;Resolution: 2.23 angstroms
C;Determination: X-ray diffraction
C;R-value: 0.234
C;Keywords: transport protein; beta-lactoglobulin; ligand binding; X-ray
crystal structure transport protein
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F;130-140/Region: helix (right hand alpha)
F;153-156/Region: helix (right hand alpha)
F;147-150,20-26,118-123,102-109,90-97,81-84/Region: beta sheet
F;66-73,54-61,41-48/Region: beta sheet
F;147-150,20-26,118-123,102-109,90-97,81-84,66-75,54-61,41-48/Region: beta
sheet
F;66-160/Disulfide bonds:
F;106-119/Disulfide bonds:
C;IDN_NRL_3D 1BSY; 2BLG; 3BLG;
C;SRCDB NRL_3D
```

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The Fragrance of Lavender

God is good.
He is colorful as
the beautiful autumn leaves,
the bright light of the sun
and the sweet fragrance of lavender
in my hand!

Indeed, I feel
God's presence,
His helping hands
touching my day
with good work, love
and results!

I am grateful
to God
and to you
who make my day
shine so bright.
Thank you!

Lan T2 Nguyen
Blindern, 12th Oct. 2007